

Lost opportunities at summit

Presidents Bush and Gorbachev enjoyed each other's company last week, and made modest progress on arms control. But by dodging doubts about the Soviet Union's future in Europe, they left the important questions unasked.

PROTESTATIONS to the contrary notwithstanding, last week's summit meeting between the Soviet Union and the United States was not a huge success. True, Presidents George Bush and Mikhail Gorbachev say they got on well together. The two governments also agreed to reduce their stocks of chemical weapons by 98 per cent; the remaining 2 per cent will be eliminated when there is general agreement that chemical weapons do not belong in modern arsenals. They also signed an agreement to agree (later this year) to reduce the number of strategic nuclear warheads deployed on either side.

That is something to be grateful for, but there are three disappointments. First, after eight years of negotiation, it is a setback that the final agreement had not been tied up by last week. Second, the reductions are less than the half originally proposed. Third, the proposed START (for 'Strategic Arms Reduction Talks') treaty is too much wrapped up in detail; the objective of warhead-reduction has been entrapped in arguments about the definition of the word 'strategic' and in qualitative wrangles about the relative merits of different kinds of strategic warheads.

There is a similar air of unreality in these side-disputes, which stem from US concern that any treaty should be acceptable to the US Senate, certain to be concerned with verification and that the mutual balance of terror should be left unchanged. (No doubt the Soviet military exerts similar pressure.) In the event, the treaty will be regarded as a permanent addition to the diplomatic landscape even though there will be more than enough strategic weapons for any conceivable purpose. (During the past eight years, improvements of accuracy have probably more than doubled the effectiveness of existing strategic arms, so that even a 50-per-cent reduction would only restore the *status quo*.)

The detailed argument on the treaty is even more unreal because of developments in the past few weeks within the Soviet Union. Is the federation of republics that constitutes the Soviet Union about to fall apart, and if so, which of its successor republics will enjoy the doubtful title to Soviet nuclear weapons? Or, to over-simplify, should Bush, the US president, have been negotiating last week with Mr Boris Yeltsin, the new president of the Russian Republic (itself a federation), rather than with Gorbachev, the president of the Soviet Union?

The word from Washington is that this delicate question was hardly touched on. That is why the summit was a

failure. There are two sets of questions that should have been tackled. One is the future pattern of European security. The other is the future of the Soviet Union as a constitutional and, more urgently, an economic entity.

The breathtaking history of the past year proves one telling point — that Gorbachev (still secretary-general of the Soviet Communist Party) has consistently acted constitutionally, allowing seven states in Eastern Europe (all of them, except Yugoslavia, members of the Warsaw Pact) to choose self-determination. There is every prospect that he would also eventually accommodate separatist tendencies in the Baltic and southern republics. Those developments have radically changed the nature of the problem of European military security as, for example, Mr Vaclav Havel, the president of Czechoslovakia, explained in his address to the US Congress last November. The common interest, now, is that there should be a moratorium on boundary changes while newly independent states win the confidence of their untried voters in new constitutions. Later, there will be more thorny problems to solve; the common interest is that that should be done constitutionally, not by armed rebellion.

Western Europe and the United States have a vital interest in the permanence of the new regime, and in particular in the continued respect of the Soviet Union for the new state of affairs. Last week's summit secured merely a reiteration that the United States believes that a united Germany should belong to the North Atlantic Treaty Organization (NATO) and that the Soviet Union disagrees. It would have been far better if Bush and Gorbachev had seriously explored the ways in which existing military forces (nuclear weapons are irrelevant) could be used for such a purpose. A merger of NATO with the Warsaw Pact would have been one device. The framework of the Helsinki Accords is another. Both may be needed. Meanwhile, the suggestion of one Soviet official last week that Moscow might apply for NATO membership is not as facetious as it seems.

The second missed opportunity concerns Gorbachev's domestic problems. It is in everybody's interest that the process of change that Gorbachev has set in train within the Soviet Union should continue, and continue to be peaceful. The perilous condition of the Soviet economy has fuelled the pent-up separatist tendencies of many Soviet republics, most vividly expressed by the election of Yeltsin as president of the Russian Federation. Gorba-



chev's personal difficulty is that he needs his Party office to secure whatever reforms may be effected by the now-elected Supreme Soviet against erosion from within. But, by continuing to hold it, he becomes tarred by the anti-Party brush. A year ago, it seemed that he might be ready to change horses, yet he seems less effective a reformer as president than he was before. The explanation is that the Party, whose economic planning is the source of the Soviet Union's economic collapse, remains also the source of other proposals for reform. And they are consistently inadequate.

Bush could not have helped resolve that problem, which is Gorbachev's, but he could have done more to help the process of economic change in the Soviet Union. The two governments signed a trade pact that will eventually assist Soviet access to US markets and US investment in the Soviet Union, but only when the Soviet Union can satisfy the US Congress on Lithuania and emigration policy. That is too long to wait, both for the worsening plight of the Soviet people and the long-term interest of the United States that US capital and companies should play a decisive part in the industrial development of the Soviet Union. There will be many in the United States who will be kicking themselves, some years from now, when it becomes plain that West Germany has been the agent of economic change in Eastern Europe and that the United States has missed the boat in its natural territory further East. But that is not the worst case, which is rather that economic chaos may provoke civil chaos and then military government in the Soviet Union, on the cards since the beginning of the year. Where would the still-incomplete START treaty then stand? □

University lottery

The new system by which British universities must bid against each other for students is a game of chance.

BRITISH universities, too often reviled by their government for their inclination to count angels on the heads of pins, are being required by their new paymaster (the Universities Funding Council, or UFC), to engage in precisely such a task — that of playing against each other in a game of poker whose rules are unknown. The game (see page 468) is that in which universities are required to say in the next few weeks what public subvention they would require to accommodate how many students in which fields of study. The only rule made public is that the cost per student must not exceed a 'guide price' set by UFC, which varies from one field to another.

UFC's objective is to win "economies of scale" — or lower ratios of teaching staff to students. Plainly these are achievable. It must always be possible to cram more bodies into lecture rooms, and the cost of operating laboratories can always be cheapened, perhaps by using graduate students as demonstrators. Universities providing individual instruction can similarly hope to

reduce marginal costs by teaching people in larger groups. That is the declared logic of the new competition, in which the marginal cost of teaching must always be zero or something like it. The other side of the coin, of course, is the quality of marginal cost teaching, with which UFC seems less concerned.

There has been some dark muttering among vice-chancellors about a secret agreement to enter bids for students at UFC's guide prices, but that must be a risk. Which university can be sure that all its potential competitors will follow the same line? But those tempted to go for growth by entering bids in chosen fields below the appropriate guide prices may find that other universities have done the same in the same fields. But those are only some of the hazards. What will happen when the bids go in, and are adjudicated? UFC will eventually decide which universities are allotted which student numbers, but will presumably make decisions in the light not merely of the cost of educating people in particular fields but of that of compensating universities for closing whole departments whose bids have been undercut elsewhere. But how will that be done, while universities are still supposedly autonomous? The bidding process may seem to universities like a poker game, but the outcome will be more like that of a lottery. □

British beef banned

Those who will not eat British beef must be prepared to settle for a European veterinary service.

THAT the West German government last week followed the French in banning imports of British beef is an offence against the spirit (and the letter) of the Treaty of Rome on which the European Communities are founded, but is no great surprise. People in France are worried by reports of bovine spongiform encephalopathy (BSE) in British cattle, and by fears that they or their cattle will thereby be contaminated. Some years ago, the British banned the import of French turkeys on the grounds that some flocks were infected by Newcastle disease virus.

British ministers are right to say that the latest affair is a test of European governments' will to carry through the single market project due to become a reality on 1 January 1993. So it is, but not quite in the way intended. The British government's case is that the spread of BSE in Britain is adequately contained by the precautions so far taken, and that "British beef is safe". Whether or not people in Britain are reassured, those elsewhere may well distrust assertions that disputed practices can be absolutely safe. Ideally, the Common Agricultural Policy (which will be spending large sums of European taxpayers' money on supporting British beef prices) would have its own veterinary service capable of reassuring all European consumers. (Instead, it has an advisory committee.) It will be a real test of the European will to be European that such arrangements would be welcome. □

Cancer risks seen in electro-magnetic fields

■ Powerlines implicated

■ Biological mechanism unknown

Boston

AFTER two years surveying the existing evidence, the US Environmental Protection Agency has concluded that there is a significant link between exposure to extremely low-frequency (ELF) electromagnetic radiation and the occurrence of human cancer. Although the report says that existing evidence is "insufficient" to prove that a conclusive causal link exists, its controversial conclusion is the US government's strongest statement so far on the potential link between human cancer and the nearly omnipresent ELF electromagnetic fields generated by powerlines, video display terminals (VDTs) and common household appliances.

A preliminary draft of the report's summary was leaked from the agency and made available to *Nature* after the report's existence was recorded in several US newspapers. A full version, entitled *An Evaluation of the Potential Carcinogenicity of Electromagnetic Fields*, is not expected to be released until later this month. The draft states that the biological mechanisms by which human cancer could be caused by non-ionizing radiation are unknown but, nevertheless, concludes that animal tests and epidemiological studies "are suggestive of a causal relationship".

In particular, the draft report says that "the several studies of leukemia, lymphoma and brain cancer in children exposed to magnetic fields from residential 60 Hz electrical power distribution systems, supported by similar findings in adults in several occupational studies also involving electrical power frequency exposures, show a consistent pattern of response". Those conclusions are sure to generate a major controversy and make it almost certain that the Environmental Protection Agency will resurrect its research on electromagnetic fields which was halted under the Reagan administration in 1986.

Controversy seems to exist within the EPA as well as outside it. According to information obtained by Louis Slesin, editor of the New-York-based trade newsletter *Microwave News*, a preliminary draft of the report recommended that electromagnetic fields such as those associated with 60 Hz electrical power distribution be classified as "probable human carcinogens", and given the agency's B-1 risk classification. This category, which denotes epidemiological evidence as well as animal evidence of a cancer link,

includes such chemical hazards as DDT, formaldehyde and PCBs.

William Farland, director of EPA's Office of Health and Environmental Assessment (OHEA) which prepared the report, acknowledged that he made the "personal decision" to delete the staff's highly controversial classification. Farland says that the uncertainty about the biological mechanism that could be involved, and the lack of a clear dose-response relationship, led him to treat electromagnetic radiation differently from a chemical hazard. The issue of classification, Farland says, "will be raised specifically" when the report is reviewed by the agency's scientific advisory board and by an external scientific review panel later this summer. The report itself, however, will be made public before the formal review and public comment period at the end of this month.

According to the report's draft summary, the "strongest evidence that there is an association between certain forms of cancer . . . and exposure to magnetic fields" comes from eight childhood cancer studies. "The consistently repeated pattern of lymphoma, leukemia and brain cancer in the childhood studies and the ruling out of several confounding exposure factors in the Savitz *et al.* study [*American Journal of Epidemiology* 128, 21; 1988] argue in favor of a causal link between these tumor types in children and exposure to ELF magnetic fields or electric fields", the report says. Also cited are "over 30 reports dealing with cancer incidence or mortality in workers in electrical and electronic occupations" carried out in Europe, New Zealand and the United States. While some of the data are conflicting, Farland stresses that the agency will "follow closely" the continuing epidemiological studies of occupational exposure to electromagnetic fields (see *Nature* 341; 374; 1989).

The EPA document does include discussion of three possible biological mechanisms for "interaction between tissue and electromagnetic fields", all of which involve the induction of weak currents and their effects on the flow of ions or ordered lipid bilayers. But it accepts that because the "relevant basic processes" are not understood, it is not possible to guess which aspect of EMF exposure might be relevant. For that reason, the report concludes that a dose-response assessment for carcinogenesis is not yet possible.

Seth Shulman

US-SOVIET SUMMIT

Seismic monitoring in favour

Washington

IN a successful conclusion to a joint experiment on nuclear test verification methods, Presidents George Bush and Mikhail Gorbachev signed agreements at their Washington summit last week that would for the first time allow seismic and 'hydrodynamic' monitoring of nuclear tests in each other's countries.

Previously, the United States had claimed that seismic monitoring was unreliable, arguing that the estimates of yields it produced depended on the geology of the surrounding rock and soil. But verification experiments made last August in the United States and last September in the Soviet Union proved to both nations' satisfaction that seismic methods can accurately detect nuclear explosions even as small as 50 kilotons.

Less technically controversial is the hydrodynamic method, known as CORRTX in the United States. Electric pulses are sent down a cable planted near the weapon, reflected back at the cable's end and measured at a safe distance. When the weapon explodes, the portion of the cable nearest it is crushed at a rate proportional to the yield, providing a measure of the explosion's force.

But CORRTX is time-consuming and expensive, and is possible only when the test is pre-announced. To monitor possible surreptitious tests, both countries have to rely on seismic networks. Allowing in-country seismic stations, which theoretically allow even unannounced tests to be detected, is expected to provide the first step towards a total test-ban treaty.

Among other scientific agreements signed at the summit is an extension of a 1973 treaty to cooperate on atomic energy research, which includes studies of the fundamental properties of matter and magnetic fusion. Additions to the original agreement include greater protection of intellectual property rights and new nuclear reactor safety initiatives based on lessons learned at Chernobyl.

A 1973 agreement to cooperate on oceanographic studies was also extended, and marine science cooperation, which had lapsed after the 1979 stationing of Soviet troops in Afghanistan, will now resume. The two leaders agreed to allow researchers to use ports in each other's countries, share research vessels and to publish joint studies openly.

Bush and Gorbachev also agreed to expand the number of undergraduate students exchanges between the two countries by 250 students (up from 750 from the United States and 250 from the Soviet Union), and to increase the target number to 1,500 students exchanged each way by 1995 or 1996 if funds are available.

G. Christopher Anderson

Students say they'll stay

Washington

ONE year after the Tiananmen Square massacre, the flood of Chinese students arriving in the United States continues unabated. But the number of exchange students returning to China has slowed to a trickle, a trend that may make it increasingly difficult for other students still in China to follow in their footsteps.

In autumn 1989, after the June demonstration, 33,000 Chinese students were studying in the United States, by far the largest contingent outside China. The total is 4,000 more than the previous year, according to the New-York-based Institute of International Education, which recently released a report on the status of Chinese students abroad. Although new students are arriving at about the same rate as last year, fewer are returning, the institute found. State Department officials say that the number of students who have returned to China since last June is only in the hundreds, most of them officially sponsored by the Chinese government or their home universities. "The historic pattern is that officially sponsored students go back and the privately sponsored ones don't", says one official. Crackdowns at home only amplify the trend, he says.

But the undiminished influx of Chinese students since the June demonstrations has come as a surprise. New Chinese regulations that restrict study abroad and more rigorous US standards for entry have undoubtedly made it more difficult for Chinese students to travel to the United States. But grassroots support for the Chinese democracy movement has spawned new private funding and boosted efforts by US universities to provide room.

Ironically, the difficulties for Chinese students begin with the problem of obtaining a US visa. About half of those applying to the US embassy in Beijing for student visas are rejected. Beyond the standard requirements, students must prove that they will have enough money to support themselves during their studies in the United States. While it was once enough to demonstrate sufficient personal or family funds, US immigration officials are now requiring more rigorous proofs of support. Cases of students who provided bogus letters listing outside sponsors are not uncommon. When such students arrive, they often reveal that the letters were written to satisfy immigration authorities and that in fact there is no independent financial support. The school must then scramble to find some sort of grant or scholarship for the student.

Most of the students who are now allowed in are those who have proven teaching or research assistantships. Such

posts are most common in science and engineering, meaning that most new visas are issued to graduate students in those areas. US academic institutions provide the largest portion of financial support to Chinese students and scholars, amounting to some \$200 million annually for teaching and research scholarships.

Once they are in, Chinese students now find it much easier to stay. Many have already taken advantage of the executive order issued by President George Bush earlier this year that allows Chinese students to remain in the United States for up to four years after their visas expire (see *Nature* 343, 402; 1990). Although the State Department requires that they officially apply for the exemption, "we're not going to deport them if they do nothing", one official says. That seems to suit the Chinese students — applications for visa extension under the executive order have been slow.

Staying in the United States feels right, too. "If you don't go back, that's a demonstration of a political statement, and most of us want to make that statement", says Liu Yuan of the Massachusetts-based China Information Center.

But what is good for Chinese students already in the United States may not be good for those who would like to come. The number of incoming students is expected to drop in 1990, in part because the current crop of students are not leaving and freeing the all-important teaching and research assistantships. Without assurance of funding, prospective exchange students are unlikely to get visas.

Meanwhile, China has instituted new rules requiring university graduates to work for five years before studying abroad, or to pay between \$530 and \$1,275 for each year of their Chinese college education (the money would be returned to them if they return to China within eight years). Another new regulation prohibits academics from travelling abroad more than once a year, a move that could hamper scientific exchanges.

The Chinese student movement outside China appears to be solidifying as its numbers increase. Chinese student groups rallied in dozens of locations around the world last week, commemorating the Tiananmen Square riots and celebrating the Chinese Democracy Movement. In the United States, large demonstrations were held in New York, where a replica of the Goddess of Democracy was set up, and in Washington, where activists and politicians criticized the Bush administration for allowing China to retain its 'most favored nations' status in trade negotiations.

G. Christopher Anderson

Small turnout in Paris

Paris

ALTHOUGH the international headquarters the Federation for Democracy in China (FDC) is in Paris, only a few hundred people braved the bad weather on Sunday evening to commemorate the anniversary of the massacre of students in Tiananmen Square. In the shadow of the Eiffel Tower, on the Esplanade des Droits de l'Homme (human rights esplanade), Chinese and Europeans lit candles in memory of the dead students, read poems and listened to speeches, including a solemn address from FDC's vice-president, Wuer Kaixi, the refugee student leader who now lives in the United States.

"This time last year", said Kaixi, "the statue of the Goddess of Democracy was smashed to the ground in Beijing. In Berlin, a few months ago, it was the wall against democracy which was smashed. Now I am standing under the Eiffel Tower, under black clouds. I am sure that the Goddess of Democracy will disperse the black clouds hanging over Beijing. As a Chinese, as a student, as a human being, I pray that the Chinese will not be forgotten. We will continue to fight."

There are about 3,000 Chinese students in France, over half of them in Paris, and an estimated 40 political refugees. Last year, an organization called the Maison Chinoise pour la Démocratie (MCD), was set up with donations from couturier Yves Saint-Laurent and others to help stranded and refugee students to obtain visas, accommodation and work (see *Nature* 340, 251; 1989).

According to Ma Tao, a young student of French literature, it is easy for students with bursaries from the Chinese government to return to China, so long as they have not been politically active. "The government encourages them", he says. But for students who have been involved with MCD or otherwise have been active, it would be dangerous to return, says Ma Tao.

The Chinese government is thought to have infiltrated student networks in France, including MCD, and to have put pressure on students. A number have been sufficiently worried to take out membership of the Chinese Communist party. According to articles in a new French language magazine, *Sinopsis*, dealing with current Chinese problems, widely read Chinese language newspapers published in Paris continue to denounce dissidents as "criminal dogs".

The small turnout for Sunday's demonstration is, says Ma Tao, a symptom of a general waning of interest in the plight of Chinese students' efforts to create political reforms. Over the past few months, the

French government, like other Western governments, has been less willing to upset Beijing. This was seen when the government refused to intervene when the radio-ship *Déesse de la Démocratie*, which set sail from La Rochelle to broadcast to mainland China, was threatened with attack by Chinese troops. The venture, organized through FDC, was finally abandoned and the ship is seeking a buyer.

"We've lost some of our enthusiasm", admits Ma Tao. "It's a difficult period for us. Now we are trying to find long-term objectives which will be useful to China." Among these is a project to establish a Chinese library in Paris to gather together documents concerning all aspects of

democracy. "Even Chinese students here have little idea how a democracy works", he says. A summer school on democracy to be held in the French Pyrenees is also scheduled, with support from the French ministry for culture and "friends in the United States". There is even a project to collaborate in the establishment of a University of Democracy in Bucharest, to be sited in the former presidential palace.

As *Nature* goes to press, there is another demonstration in Paris. "Flowers against tanks" are being laid on the steps of the Chinese Embassy. "We hope the whole world will not forget what happened in China", says Ma Tao.

Peter Coles

BIODIVERSITY

Preserving the present

Sydney

THE Centre for Genetic Resources and Heritage (CGRH), a library that will be filled with genetic material from rare or endangered Australian plants and animals, has been set up at the University of Queensland, but lack of federal government support is forcing the library to seek support from commercial interests in Australia and overseas.

CGRH, part of the University's Centre for Molecular Biology and Biotechnology, will be a "genetic Louvre", in the words of director John Mattick. Collected material will be stored as cryogenically or desiccated cells and tissue, isolated DNA and cloned gene libraries. Other gene resource libraries have traditionally restricted themselves to collections of microbiological cultures and seed material. According to Daryl Edmondson, coordinator of the gene library, the CGRH is unique in that it will "actively collect data. Most other libraries simply collate their own collections." CGRH will collect cells or DNA from Australia's most threatened species, such as the false water rat, one of the country's rarest mammals, and the short-necked swamp tortoise. The university's large collection of bacteria, fungi, yeast, algae and viruses, accumulated over 25 years, will also be represented at the centre.

CGRH, at present supported by the university, is seeking help from private sources and has been in touch with corporations in Australia and Japan and scientific foundations in the United States. Mattick complains that the Australian government "can't see the benefit of collecting genetic resources and eventually sequencing key genomes". The project will not yield any visible benefit for ten years, according to Mattick, but, if it is not done, "subsequent generations will see we had the technology to keep (DNA) software and will ask why we didn't do it".

Tania Ewing

INDIAN SCIENCE

Talent down the drain

New Delhi

SOME 324,000 engineers, doctors and scientists from India have so far migrated and the figure may exceed half a million by the year 2000 according to a study carried out by the New-Delhi-based Centre for Planning, Research and Action at the request of the Department of Science and Technology.

There has been tremendous increase in the magnitude of the brain drain since 1961. Only 8,900 skilled professionals moved abroad in the period 1962–65. By 1982–85 the number had reached 84,200.

One-third of engineers and technologists emigrate, as do 28 per cent of doctors and 22 per cent of scientists. Highly qualified scientists and technicians make up 20 per cent of the Indian work force abroad. The report calculates that as a result India lost benefits by 1985 equivalent to \$8,000 million to the United States, \$2,600 million to Canada and \$2,200 million to the United Kingdom.

The study has attempted to project the size of the brain drain in the future after taking into account recent restrictions on emigration. The number of Indians living in countries outside the subcontinent is estimated at 475,000 people in 1995 and 539,000 in 2000.

According to the projections some 61,400 skilled workers will emigrate in the period 1988–2001. Of these, 1.5 per cent will go to the United Kingdom, 12 per cent to Canada, 26 per cent to the United States and the largest number, 28 per cent, to West Asia.

Economic factors, inadequate attention by Indian industry to research and development, political and historical factors, prestige attached to living abroad and immigration policies that give preference to skilled manpower are some of the causes of the brain drain, according to the study.

K. S. Jayaraman

X-RAY ASTRONOMY

New telescope hits orbit

Munich & Washington

ROSAT, the multi-nation Roentgen satellite, was launched successfully last week, ready for a six-month survey of cosmic X-ray sources and a year or more of detailed study of black holes, very hot stars and supernova gas clouds that emit X-ray radiation.

Although the ground controller at Cape Canaveral briefly lost radio contact with the satellite after its launch on a Delta II rocket, full communications have resumed. The satellite extended its solar panels and other appendages without trouble, and is transmitting a "strong signal", according to Goddard Space Flight Center, the US laboratory that oversaw the launch. First scientific data should arrive today when Rosat open its camera door, followed by two months of calibration.

Rosat has two telescopes on board that will scan the sky in the ultraviolet and X-ray frequency ranges. In the 18 months it is expected to function, Rosat should transmit enough data to create a sky atlas of X-ray sources with over 100,000 entries. At the same time, researchers will use Rosat to study the X-ray background radiation from the sky.

Boasting the smoothest mirror in the world, Rosat is one hundred times as sensitive as any other X-ray telescope yet launched. Coated with a fine film of gold, the mirror (made by Carl Zeiss) varies from perfect smoothness by only 10^{-8} cm, close to the dimensions of an atom.

Rosat is an international project with three partners, West Germany, Britain and the United States. The main body of Rosat and its mirror was built in West Germany beginning in 1982 by three companies — Messerschmitt-Bölkow-Blohm, Carl Zeiss and Telefunken Systemtechnik — as well as the Max Planck Institute for Extraterrestrial Physics and the West German Aerospace Research Establishment (DLR).

West Germany contributed the larger X-ray telescope, with an aperture of 84 cm, and two of its three photodetectors. The third was provided by the United States. Britain contributed a lower-resolution wide-angle telescope of 57 cm aperture to study ultraviolet radiation. The satellite cost DM560 million (\$333 million).

Rosat was originally scheduled for launch on the US space shuttle in 1987. But after the Challenger explosion, the launch date was pushed back so far — to 1994 or possibly even later — that project leader Joachim Trümper, backed by the West German government, sought another way to get Rosat into orbit. The United States finally agreed in early 1987 to provide a Delta II rocket (see *Nature* 326, 820; 1987).

Steven Dickman

& G. Christopher Anderson

Where there's no will . . .

Munich

FOUR weeks before monetary union removes most of the remaining barriers between East and West Germany, there are few plans and little money to tackle the problem of providing education to East German students without damaging West German universities. East German universities, after 40 years of adherence to Marxism-Leninism, have no one to teach the Western-style law and economics that will soon overtake the country, and few scientists seem willing to sacrifice careers in the West to help their new-found compatriots.

One suggestion, put forward by the organization of university teachers (Hochschulverband, HSV) at its 40th annual meeting in Frankfurt on 25 May, is that West German professors should visit East Germany *en masse* and teach there half-time. HSV urged the *Länder*, which run universities in West Germany, to free professors from half of their teaching duties, starting as early as this autumn. 'Flying faculties' could then be set up containing a mixture of university professors (50 per cent), retired professors (25 per cent) and outside lecturers (*Privat-Dozent*) with a university teaching qualification but no position at a university.

HSV is also collecting money to endow chairs at East German universities to be awarded to professors who suffered discrimination at the hands of the former Communist regime.

If enacted, the plan would be the first concerted effort to rehabilitate East German universities, where little has changed since the fall of the Berlin Wall and the Communist government (see *Nature* 344, 605; 1990). Only a few West German professors have volunteered to teach in East Germany for more than a few days, or at most for a summer, and university partnerships have sprung up only sporadically.

HSV spokesman Jürgen Janik says the idea arose out of discussions with East German professors, many of whom are joining HSV, and that there have already been more than 20 volunteers for the programme in law alone, enough to create the first flying faculty at either Erfurt or Leipzig in time for the winter term, which begins in October.

The proposal received general endorsement from one prominent *Länder* politician, Minister-President Walter Wallmann of Hesse. But Wallmann would not say how much money Hesse would provide, nor where new teachers would be found to take up the teaching load at Hesse's universities. And representatives of other *Länder* such as North Rhine-Westphalia (NRW), which has the largest population and the most universities, criticized the

proposal as too vague and predicted that West Germany could not afford it. "Let's see in three weeks if Herr Wallmann still supports it", said NRW education ministry spokesman Michael Schmidt.

Sharper criticism came from the chairman of the influential Wissenschaftsrat (science council), Dieter Simon, himself a university professor and legal scholar. Advising politicians not to support the plan, he said "a politician earns his reputation by being a realist, and any realist would be sceptical about this idea". Simon is in close touch with the federal and *Länder* governments, which each send representatives to sit on the Wissenschaftsrat.

Simon said that the plan would fail to achieve its stated goal of preventing a rush of East German students to West Germany's already overcrowded universities. Short-term visits would be inadequate: "if you want to teach West German law, you have to have six [professors] there all the time", he said.

Simon also believes that those who volunteer to go the East "are not likely to be of the highest quality", and says "there is no sense in sending [lecturers to East Germany] whom no one wants to hear [in the West]". He added, "You have to send the best — not like Fritz [Frederick the Great] who found teachers for the Prussian primary schools by hiring sergeants who had been drummed out of the army." The fields in which East Germany needs the most help, according to Simon, are exactly those in which there is a shortage of teachers in West Germany.

Even former East German professors who are being offered their old jobs back by apologetic university administrators are not flooding back to East Germany to take up the challenge. Biochemist Peter Bohley of the University of Tübingen has been invited back to the University of Halle, where he used to teach and says he "would love to go". But only for two weeks, he added, because he cannot leave his research in West Germany.

The prospect of help for East German universities is also dimmed by the reluctance of the West German government to help its own universities. A DM6,000 million (\$3,570 million) programme proposed by West German Education Minister Jürgen Möllemann to keep young researchers at universities for the next 5–10 years until permanent jobs for them become available has been postponed repeatedly.

But Simon has no counterproposal to replace the HSV plan should it indeed fail. "I have not heard a single reasonable suggestion" for helping East German universities back to their feet, he asserted.

Steven Dickman

Genome use for computer

Tokyo

JAPAN's fifth-generation computer project has found its first international application in an agreement, announced last week, to work with the US Argonne National Laboratory to help analyse the flood of data generated in the human genome project. Under the agreement, Japanese and US scientists will jointly use computer systems from the fifth-generation project to develop techniques for handling human genome data.

The fifth-generation project is due to end next fiscal year, a decade and ¥50,000 million (\$325 million) after its promises of a computing revolution shook foreign governments into starting artificial intelligence programs of their own. The revolution never arrived, but Japan was left with new international standing in computer science — and an impressive set of hardware and software for parallel computing.

This week, two of the latest personal sequential inference machines, the PSI-II, will be delivered from the fifth-generation project's Institute of New Generation Computer Technology (ICOT) to Argonne along with supporting software and a database management system, called Kappa. The PSI-II machines, worth about ¥10 million (\$65,000) each, will be loaned to Argonne free of charge.

Using a high-capacity computer network link via Hawaii, Argonne researchers will be able to access ICOT's huge parallel computer, the Multi-PSI, in Tokyo to run programs. Similarly, ICOT researchers will be able to access parallel processors at Argonne's Advanced Computer Research Facility. US researchers elsewhere will be able to gain access to ICOT's machines by setting up an account with Argonne.

Apart from using the Japanese computers, the US-Japanese team will investigate the use of other computer systems to handle genome data, including shared-memory processors (such as the Sequent, Encore and BBN machines at Argonne), multicomputers like that developed by Intel, and networks of workstations.

Researchers at Argonne and ICOT share the view that logic programming and parallel processing provide an ideal framework for the rapid computational analysis and storage of human genome data. Shunichi Uchida of ICOT says that ICOT's extended relational database management system Kappa, originally designed for handling information from dictionaries, is particularly suited to human genome data because both dictionaries and genome databases contain "nested" structures of "explanations within ex-

planations", which are hard to handle on conventional database systems. ICOT have already loaded some GenBank databases into Kappa by accessing the DNA Data Bank of Japan (DDBJ) in Mishima.

Ross Overbeek, senior scientist at Argonne, says that in the next few months the US-Japanese team will select a small number of specific projects as prototypes to demonstrate the significance of the emerging computer technology for the human genome project.

At first, only about ten researchers (four or five on each side) will be directly involved. But in future Overbeek says the team hope to form a much wider collaboration involving US and Japanese biologists.

As examples, Overbeek points out that Argonne is establishing a centre for analysis of ribosomal sequence data with Carl Woese's group at the University of Illinois. The laboratory is also working on the integration of data from multiple biological databases with Toni Kazic of

Washington University.

ICOT researchers are finding it much harder to find Japanese biologists with whom to cooperate. Uchida says each Japanese biologist seems to have his own philosophy on computer use and he has found only three or four people who have shown an interest in the application of logic programming to genome data. But among his allies are researchers at DDBJ and people involved in drawing up plans for a human genome project for the Ministry of Education, Science and Culture.

The Argonne-ICOT project will initially run until March 1992 when the fifth-generation computer project is scheduled to end. Kazuhiro Fuchi, director of ICOT, says that MITI has only just begun discussing the future of ICOT. One possibility is to close the institute, or it could be continued in a "new style", he says.

And Fuchi is clearly encouraging Uchida and his co-workers at ICOT to develop the collaboration with Argonne into something bigger. **David Swinbanks**

SUPERCONDUCTING SUPER COLLIDER

An offer Japan can refuse

Tokyo

In an attempt to tease from the Japanese government its help in building the Superconducting Super Collider (SSC), a team from the US Department of Energy (DOE) last week suggested that Japanese industry might be allowed to build as many as half of the accelerator's 10,000 superconducting magnets in return for a contribution to the project's \$8,000 million construction costs. But the offer failed to elicit any firm commitment from Japan, and is not likely to go down well with the US Congress either.

The superconducting magnets were on a list of nine items shown to representatives of the Science and Technology Agency (STA), the Ministry of Education, Science and Culture (MESC), the Ministry of Foreign Affairs and the Ministry of International Trade and Industry (MITI) as examples of technologically advanced devices that Japan might be interested in providing. Also included was equipment for the SSC's particle detectors, whose billion-dollar cost is not included in the \$8,000 million price. At a press conference on 4 June, DOE officials refused to reveal details of the list, but it was seen by a member of the Japanese press who passed the information onto *Nature*.

But the technological items that might attract Japanese interest are the same ones whose manufacture the US Congress has insisted, so far, should remain in the United States (see *Nature* 345, 375; 1990). Nevertheless, Henson Moore, DOE deputy secretary and head of the US dele-

gation, said "should the Japanese government decide it would like to contribute by providing the magnets, Congress would support such a move".

No quick decision can be expected from the Japanese government because of wrangling between the various ministries and agencies involved and because of limitations on their budgets.

Traditionally MESC has been in charge of Japan's major high-energy physics projects, such as the positron-electron collider TRISTAN in Tsukuba. But recently, STA has begun to encroach on MESC territory with plans to build a huge 8 GeV synchrotron near Osaka.

Toichi Sakata of STA, who met the US delegation, stresses that the SSC should fall under the responsibility of MESC for "historical" reasons. But Yubun Narita, director of scientific affairs at the Ministry of Foreign Affairs, says that no decision has been made on which government agency should take the lead.

For a variety of reasons, MESC is most unlikely to come forward with the sort of money (about \$1,600 million) that DOE is seeking from foreign partners. The ministry is very conservative and has no precedent for contributing such large sums of money to a foreign project. In addition, Japanese high-energy physicists affiliated to MESC are divided in their opinions about the SSC.

Some researchers at the High Energy Physics Laboratory (KEK) in Tsukuba are collaborating with their US colleagues in drawing up plans for the SSC. But others, at KEK and at Tokyo, Kyoto and Nagoya

CHERNOBYL

Still too hot to touch?

Munich

AN international team of experts is to visit the area around Chernobyl to assess ill-effects on the health of the local population and environment from radiation released in the 1986 nuclear accident. The investigation, announced by the International Atomic Energy Agency (IAEA) last month, is a response to a Soviet request to IAEA made in October 1989, and will also evaluate the measures taken by the Soviet government to cope with contamination.

IAEA will report late in 1990. The Food and Agricultural Organization, the European Commission, the United Nations Scientific Committee on the Effects of Atomic Radiation and the World Health Organization are helping with the study.

Severe malformations of local flora and fauna have been blamed on radiation from Chernobyl, but these anecdotal reports do not accord with what is known of the after-effects of the Hiroshima and Nagasaki explosions. **Steven Dickman**

universities, have no intention of actively participating.

In particular, Tokyo University researchers would prefer to continue their collaboration with Europe on the OPAL experiment on the LEP electron-positron collider at CERN and on CERN's Large Hadron Collider (LHC) — if it is built.

Furthermore, many high-energy physicists want MESC to fund a huge linear collider, the Japan Linear Collider (JLC), which would have many of the same scientific goals as the SSC (see *Nature* 344, 8; 1990). The proposed JLC is an electron-positron collider, unlike the SSC which will collide protons, and although it is of lower energy than the SSC, Japanese scientists believe the JLC will provide cleaner and superior results. But if MESC were to contribute to the construction costs of the SSC, the JLC project would be in jeopardy.

Nor does it seem likely that STA could fund SSC construction because any move to make STA the lead agency is likely to be opposed by MESC-affiliated physicists who want to participate in the experimental phase of the SSC.

Despite all these problems, Henson Moore says that after his discussions with the various ministries and agencies he is "optimistic" that Japan will join the project, because he says Japanese participation would give meaning to the recent agreement between Prime Minister Toshiki Kaifu and President George Bush for the two countries to form a "global partnership".

Discussion of Japanese participation is likely to move forward only if it is taken up at the highest level of the Japanese government. **David Swinbanks**

Bids will exceed funds

London

If the public statements of university vice-chancellors are to be believed, then the total bids for undergraduate teaching funding submitted to the Universities Funding Council (UFC) in the next two weeks seem certain to exceed the available resources.

For the first time, student places for 1991–92 will not simply be allocated to British universities. By 22 June this year, each university must tell the UFC how many students it wishes to teach in each subject, and at what price each student can be taught (see *Nature* 342, 843; 1989). Except under "exceptional circumstances", these bids must not exceed 'guide prices' for each subject area, calculated by the UFC.

The idea behind the system is that universities taking on more students can make 'economies of scale', and bid at lower offer prices. By forcing the universities into competition for students, government policy to increase participation in higher education could be met at a lower cost per student.

But this is rejected by the vice-chancellors, who maintain that even the guide prices are too low. In public, vice-chancellors say they will bid for existing student numbers at the guide prices, in effect operating a cartel (although there is no formal agreement) which could defeat the UFC's purpose. Even for student places above their present numbers, most vice-chancellors say they can afford to bid only at, or just below, the guide prices.

Sir Peter Swinnerton-Dyer, chief executive of the UFC, thinks it unlikely that the vice-chancellors will maintain a unified front, because a cost-effective university able to bid for increased student numbers at competitive prices would gain under the bidding system. There are few hints at competitive bids, although Professor John Ashworth, vice-chancellor of the University of Salford (shortly to move to the London School of Economics), is quick to point out that vice-chancellors' public statements on guide price bids refer only to existing student places. He says that Salford, which he describes as "one of the most cost-effective universities in the country", could take up to 40 per cent more students by the mid-1990s.

But if most bids do come in at the guide price, this would need a substantial increase in the UFC's funds from government in the November public expenditure statement, as most universities are planning expansion. Few vice-chancellors expect Lord Chilver, chairman of the UFC, to fight hard for funds to match the bids. Sir Graham Hills, vice-chancellor of the University of Strathclyde, describes

the present situation as "farcical", and believes that the UFC will simply impose increased student numbers on universities at some "fictional price".

This prospect angers most vice-chancellors. Clark Brundin, from the University of Warwick, says that universities have prepared detailed plans for student numbers and finance up to 1994–95, to accompany their bids, and it would be irresponsible of the UFC to ignore these.

Whatever the outcome next February, when the UFC announces its decisions for student places and funding for 1991–92, most observers agree that the UFC will respond to 'overbidding' by universities by reducing the value in real terms of the guide prices in subsequent years. Swinnerton-Dyer says that the UFC has not yet decided how to link guide prices to inflation.

HUMAN GENE THERAPY

Approval next time round?

Washington

THE chance of the first human gene therapy experiment winning approval improved last week when the US National Institutes of Health human gene therapy subcommittee conditionally approved an experiment to treat adenosine deaminase (ADA) deficient severe combined immunodeficiency. If its provisos are met, full approval could be given at the next meeting on 30 July.

The experiment hopes to correct the ADA deficiency by inserting a functional human ADA gene into T lymphocytes taken from the patient and readministering the ADA-corrected T cells back to the patient (see *Nature* 344, 483; 5 April 1990). The experiment was subject to tough scrutiny at the previous meeting, partly because ADA deficiency is very rare, with less than one in a million births affected, and so the pool of potential patients is very small, and partly because the risks and benefits of the experiment must be weighed against existing therapies provided by bone-marrow transplantation and enzyme replacement with the recently approved drug PEG-ADA.

As bone-marrow transplantation is the preferred method of treatment where a sibling-matched donor can be found, patients will be enrolled in the experiment only if they are considered unsuitable for bone-marrow transplantation. In addition, patients must be at least one year old and have been receiving PEG-ADA for a minimum of nine months so that researchers can determine the extent of immune reconstitution achieved with PEG-ADA alone.

Faced with declining government funds per student, vice-chancellors are looking at alternative systems. With the Committee of Directors of Polytechnics, the Committee of Vice-Chancellors and Principals has set up a working party to look at funding mechanisms for higher education, which meets for the first time this week.

Professor Gerry Fowler, rector of the Polytechnic of East London and a member of the working party, believes the entire system of higher education funding is "up for grabs" over the next three years. Many university vice-chancellors would like to see the cost of an expansion of higher education met in full by government. But radicals such as Hills see direct financial links between universities and government as harmful. Hills wants teaching costs switched to fees paid by students, with any government support through scholarships or vouchers, and rejects the claims of many of his colleagues that this would deter children from poorer families.

Peter Aldhous

Concerns were raised by subcommittee members at the previous meeting about the withdrawal of PEG-ADA in phase three of the planned experiment. In the hope of making the experiment more acceptable to reviewers this time round, principal researcher R. Michael Blaese of the National Cancer Institute and his associate W. French Anderson of the National Heart Lung and Blood Institute presented the subcommittee with a substantially modified experiment from which phase three had been dropped.

There had also been criticism of the lack of pre-clinical data. Countering this, Blaese and Anderson presented a brief summary of experimental studies carried out by collaborative researcher Claudio Bordignon in Milan. Bordignon has shown that ADA gene-corrected T lymphocytes were able to survive and function in immunodeficient mice. Subcommittee member William N. Kelley pointed out that this is "one piece of evidence that suggests having the gene [for ADA] intracellularly is an advantage". But subcommittee members were not convinced and asked to see supporting data. It remains to be seen whether they will then be prepared to extrapolate Bordignon's research to the proposed gene therapy experiment.

Blaese says he has already met and discussed the risks of treatment with three families with ADA-deficient children. Blaese believes that "by studying three or four patients, we are going to know whether this therapy is worthwhile and whether it has a chance of working".

Diane Gershon

SEISMOLOGY

Earthquake rocks Romania

London

THE earthquake that hit Romania on 30 May was, at magnitude 6.9, similar in severity to the 1988 Soviet Armenian earthquake, but the nine fatalities reported so far are much fewer than the tens of thousands killed in Armenia. The low Romanian death toll is partly because the earthquake was deep — the epicentre was about 90 km below the surface — and partly because most of the fragile buildings in the affected area had been demolished by a 7.2 magnitude earthquake in 1977.

The Romanian earthquake, classified as 'intermediate depth', generated shock waves over a wide area and was felt about 1,300 km away in Moscow, but caused less ground movement at the surface than the shallow 1988 Armenian event, whose epicentre was only 3 km down. At 45.75° N and 26.65° E, the earthquake was centred in an area called the Vrancea focus, from which the Carpathian Mountains extend in an arc to the north and the west.

According to Professor Dan McKenzie

EUROPEAN SCIENCE

Eureka finds Eastern Europe

Munich

WESTERN European industry ministers meeting in Rome last week announced that they would extend a hand to Eastern European countries interested in participating in the Eureka research and development programme. Eastern European states will not be able to join Eureka, but their involvement will be encouraged in both the planning and the execution of joint projects. Until now, Eastern European countries have been able to join Eureka projects only when they have begun.

The membership of Eureka, a flexible programme that coordinates multinational research and applied science projects in Europe, now includes Scandinavia, Austria, Switzerland, Iceland and Turkey in addition to the 12 European Communities nations. Although Yugoslavia had an earlier application to join turned down, it and two other Eastern European states, Hungary and the Soviet Union, already participate in 9 Eureka projects. East Germany is expected this year to join EUROTRAC, a project to study the path of air pollutants through Europe, and EUOMARBLE, which is concerned with the conservation of historical landmarks.

One hundred new projects were taken up by Eureka last year, making a total of about 400. Member states and companies now contribute DM16,000 million (\$9,500 million), up from DM 13,000 million a year ago.

Steven Dickman

of the University of Cambridge, the earthquake originated in a section of oceanic plate, which is slowly sinking into the mantle. This 'subduction' occurs when oceanic plate is pushed towards a continental plate and burrows underneath.

Subduction in the Carpathian Mountains may now have finished, but a slab of rock has been left protruding almost vertically downwards to a depth of about 100 km. Last week's earthquake probably resulted from part of the leading edge of the slab shearing off along an oblique fault line.

The Vrancea region has a history of earthquakes. Over two thousand people died in the March 1977 tremor, which was twice as powerful as the latest one, but Professor Nick Ambraseys of Imperial College, London, says that the lower death toll this time was not simply due to the difference in magnitude. In 1977, most deaths were in Bucharest, where old multi-storey concrete buildings, many of which had been weakened by a previous earthquake in 1940, collapsed. Very few of these vulnerable buildings survived the 1977 earthquake to cause problems last week. Poor building standards were also blamed for the immense loss of life in the 1988 Armenian

earthquake.

William Spence, of the US National Earthquake Information Center, describes the Romanian earthquake and a smaller earthquake the previous day in Northern Peru as "moderate sized" events. The Peruvian earthquake was of magnitude 6.3 but the death toll ran into the hundreds, with mudslides a major hazard. Although the Romanian and



Map of the earthquake area. The slanting rectangle in the map inset marks the approximate position of the subducted oceanic plate, descending into the mantle.

Peruvian earthquakes attracted considerable press attention, Spence says both were dwarfed by a 7.5 magnitude earthquake in a remote area of Sudan, in the African rift valley, on 20 May.

Peter Aldhous

NON-PROLIFERATION TREATY

Brazilian atomic bomb now a possibility

São Paulo

ONCE a new uranium-enriching centre is complete, all it would take for Brazil to build an atomic bomb is \$2 million and "seven days", according to a report from the Brazilian Physics Society. The report, complete with a sketch of a bomb employing 18 kg of enriched uranium, caused some embarrassment as it was presented by the society to the Secretary of Science and Technology, José Goldemberg, at the same time as the US representative to the International Atomic Energy Agency, Richard Kennedy, was touring Brazil.

The uranium enriching unit is being built by the navy to provide fuel for a submarine reactor. Although the Brazilian constitution requires that nuclear energy be used only for peaceful purposes, Kennedy urged Brazil to ratify the Nuclear Non-Proliferation Treaty. He said that the constitution is for "internal use", and that Brazil could

show the world its peaceful intentions by signing the non-proliferation treaty.

Brazilian diplomats countered by describing the treaty as "discriminatory". Only if the treaty is changed to improve the exchange of technology between the developed and the developing countries at its next scheduled revision in August would they be willing to sign. The president of the Brazilian National Commission for Nuclear Energy (CNEN), José Luiz de Santana Carvelho, echoed diplomats in denouncing "pressures" for the country to sign and even revived talk of "economic neocolonialism".

Kennedy said that exchange of technology with the United States will not proceed if Brazil does not ratify the treaty, and went on to criticize Brazil's military relations with Iraq. Brazil has long been a major arms exporter to Iraq, selling large quantities of armoured cars and artillery rockets.

Ricardo Bonalume

Gaia metaphor unfalsifiable

SIR—Jim Lovelock's nice-guy image may not last if he persists in responding to his critics by impugning their motives. Biologists, he says, "seem to see Gaia as another threat to the sanctity of their creature, neo-darwinism". Reductionists, he says, are engaged in a "dogmatic crusade". Lovelock apparently even sees fit to accuse me of a witch hunt: "like some figure of the Inquisition, he publicly burned several imaginary Gaias, and his pyrotechnic demolition of the strong Gaia stole the show. But when the sparks faded, the real system Gaia was still there hidden only by the smoke"¹.

Lovelock neglects to note that my criticism is available in the literature². Others may judge for themselves whether the Gaias I address, drawn verbatim from Lovelock's publications between 1974 and 1988, are "imaginary" or not.

Now, has the "real system Gaia" (Lovelock's most recent Gaia, also called "geophysiology") gone up in flames? Lovelock insists that "Gaia theory is testable", and lists some of the research spawned by the Gaia metaphor¹. The investigations Lovelock cites, however, are generally not tests of the Gaia hypothesis, because "geophysiology" is unfalsifiably vague; negative results can be explained away simply by reinterpreting the theory.

For example, in 1987 Lovelock proposed that algae act as a global thermostat, producing dimethyl sulphide (DMS), a precursor of cloud condensation nuclei, to try to cool the Earth when it is warm³. Unfortunately, ice-core data published the next year suggested exactly the opposite. DMS levels are apparently highest during glacial periods; if the algae are important at all, they act to make the Earth even colder when it is cold⁴. Undeterred, Lovelock promptly inverted the theory to match the data. He now argues that Gaia actually *prefers* the glacial deep freeze, and that interglacial periods represent "a fevered state of the planet"^{1,5}. How could anyone prove or disprove a "hypothesis" so ambiguous that it can predict one result, but also explain the exact opposite? And what could one *do* with it? Basing specific policy advice on such a fuzzy theory seems irresponsible.

It is ironic that Lovelock says Gaia "could be crucial to understanding the consequences of pollution and environmental disturbance"¹. In the 1970s, Lovelock himself used Gaia to argue *against* controlling chlorofluorocarbon pollution of the atmosphere, saying "We tend to forget that pollution is a way of life of many natural species . . . Our capacity to pollute on a planetary scale seems rather trivial by comparison, and the system does seem to be robust and capable of withstanding major perturbations"⁶. He now

admits he may have been wrong, although he still sees fit to accuse his opponents from the 1970s of "global hypochondria"⁵.

Better understanding and preserving the global biogeochemical system will require a lot of difficult, important science, and we should get on with it. I hope that the Gaia metaphor will inspire interesting research into how that system works. We should be careful, however, not to pretend Gaia is a testable hypothesis, much less a basis for managing the biosphere. The risk is this: a metaphor like Gaia, flexible enough to be wrapped around almost any data set, is also versatile enough to be invoked, *ad hoc*, to lend a spurious air of scientific legitimacy to almost any reckless conjecture.

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Electronic journals

SIR—John Maddox in his News and Views article "Towards the electronic journal" (*Nature* **344**, 287; 1990) raised some interesting issues. In passing, a removable 45-megabyte hard-disk cartridge costs about £100, not \$1,000, and weighs as much as a copy or two of *Nature*. So one issue of *Nature* could comfortably sit within a megabyte, and the means to provide a year's issues on disk are already with us, magneto-optical disk technology notwithstanding.

Earlier this year, Sony announced two new products (neither of which will be commercially available for at least six months) both of which may cause a revolution in CD-ROM technology. The first is a rewritable CD system, in itself not new, but this system should be only marginally more expensive than present CD players. It could easily be imagined that a six-pack CD peripheral could provide cheap, removable, on-line storage of 1.5–3 gigabytes at a fraction of the cost of present hard-disk systems, and without the usual fragility of floppy and hard disks. Transfer of the technology to video disks could allow storage of, say, 14 gigabytes.

The second product is an electronic book, based on a CD-ROM, with an LCD

viewer. Each disk would offer 256 megabytes of storage, equivalent to about five years of *Nature*. As an alternative to LCD, Reflection Technology's Private Eye wearable viewing system offers a reasonable quality image (up to 1,024 × 280 pixels), and twin systems would allow three-dimensional imaging. Sony has not estimated a cost for the electronic book, but the alternative viewing system would start from only about \$100.

The CD revolution offers many possibilities. A combination of ROM and RAM equivalents on CD could provide an unerasable kernel of data with an appendix of either specialist terms or updates, for example, that could be rewritten by the user. Transmission of live video down an ordinary telephone line using compression techniques derived from fractal geometry offers the opportunity to transmit huge quantities of data in a very short space of time. Subscribers to *Nature* might be able to receive the week's issue, detailed photographs and all, by telephone.

What is more, CD-based literature can be immensely more informative than its paper-bound predecessor. Not only can typefaces be varied to suit the reader, but animated images can be readily provided. What remains to be seen is the power of the various forms of inertia likely to delay the implementation of these items. There are undoubtedly as many, if not more, interested parties keen to impede such changes as there are to produce them. It will be interesting to observe the outcome of the various skirmishes, and to see just how long it is before *Nature* is offered in CD form. If nothing else, CDs are considerably lighter and cheaper to post than paper counterparts.

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Two too many

SIR—Some in Great Britain are apparently misinformed about developments in the former colonies.

John Maddox (*Nature* 8 March)¹ identifies New Hampshire as "the state which is fiftieth (out of 52) in size," yet authoritative sources² list only 50 states, of which New Hampshire currently ranks 44th in area and 39th in population. As Maddox implies, substantial growth beyond the original 13 colonies has indeed occurred, although the growth has been less than suggested.

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The curious case of *Acarus crossii*

James A. Secord

One hundred and fifty years ago, life was created by electricity — or so it was widely reported. The story illustrates the power of the press, and brings to mind more recent episodes in science.

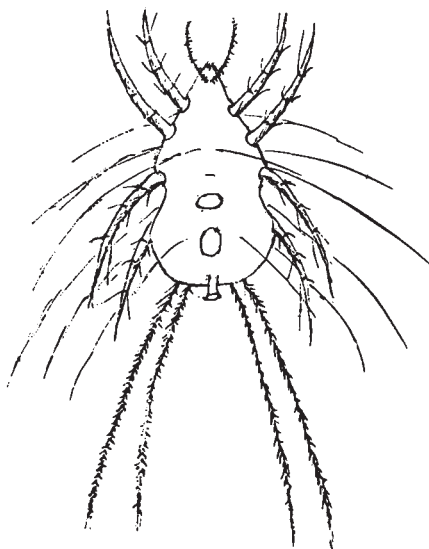
A FEW months before Victoria ascended the throne, living mites of the genus *Acarus* unexpectedly crawled out of an electrical experiment in a private laboratory in Somerset. The experiment was conducted by Andrew Crosse, a wealthy English gentleman and avid natural philosopher. Publication (without his permission) on the last day of 1836 in a local newspaper under the headline "Extraordinary Experiment" led to an international sensation. It was claimed that life had been created, and Crosse was accused of being a Frankenstein, a "disturber of the peace of families", "a reviler of our holy religion". By others, he was hailed as a genius who had broken the boundary between life and matter. For some, the discovery represented the foundation of a new discipline. For others, it was a stepping stone to atheism, an editorial coup, or a joke.

In retrospect, the electrical creation of mites is easy to dismiss as absurd. But by taking the episode as seriously as the Victorians did we can use it to witness the origins of our own troubled relationship between scientists and the public. The story has many contemporary resonances. For 'popular science' in a recognizably modern form was created in the early nineteenth century, as a result of larger changes associated with the reaction to the French Revolution and the early stages of industrialization. The institutions, ideas and instruments of science were transformed and the audience for science altered almost beyond recognition.

Andrew Crosse, born in 1785, made his unexpected discovery while attempting to form artificial crystals from silica. Working in a laboratory in the music hall of his Jacobean country house, he had been fascinated by possibilities of the voltaic cell, and hoped to use it to imitate the formation of crystalline deposits in nature. A dilute solution of silicate was set dripping over a porous stone electrified by a small battery. At first everything went as planned. After the first 14 days "a few small whitish excrescences or nipples" were visible. Four days later, these had enlarged, and filaments had appeared. By the twenty-second day, they had enlarged still further; four days more, and "each figure assumed the form of a perfect

insect". Crosse claimed that only then did he suspect that something more than "an incipient mineral formation" was being produced. When that "mineral formation" wriggled its legs on the twenty-eighth day, Crosse was understandably "not a little astonished".

The first stage in the story had depended on the voltaic cell, one of the most important of the new experimental technologies. The second stage depended



Crosse's drawing of "one of the insects formed under the influence of voltaic electricity", as seen under the microscope. The drawing was sent to Richard Owen, the eminent comparative anatomist, in April 1837.

on the steam press and other new printing methods, innovations of the Industrial Revolution. For the first time, newspapers, periodicals and books began to be cheap enough to be bought by a much wider readership among the middle classes and educated artisans. The technology of print, however, was rarely controlled by those who wielded the technology of experiment. The demarcated roles for journalists and men of science which had developed around 1800 meant that the public received its knowledge through channels almost entirely distinct from those employed by specialists. Producers of science and their publics stood in a new and more distant relation.

The *élite* gentlemen of science became involved in many attempts to present a sanitized, safe image of science to the mass audience, most notably at the British Association for the Advancement of Science. This image had no place for

experiments on the origin of life, but the Association's leaders unwittingly played a key role in thrusting the acari into the public eye. At the Bristol meeting in 1836, several months before his experiments produced insects, the reclusive Crosse was brought forward and hailed as a genius whose work on crystals had the potential for revolutionizing geology.

Because of this publicity, Crosse's name became well enough known to sell newspapers, which is how his mites initially escaped into the public domain. The editor of the *Somerset County Gazette*, which commenced publication at the very end of 1836, learned that insects were emerging from Crosse's apparatus, and concluded that a page three feature in the first issue would attract readers. In a season of slow news, the announcement was greedily taken up, first in *The Times* and then in hundreds of other newspapers, magazines and journals throughout the world. The power of the press was released in a full tide. For most readers the experimental creation of life was a scientific fact.

But the insects had only begun to make their way into science. For this to happen, the experiment had to be transferred out of Crosse's country house (or, more accurately, out of the newspapers) and into other laboratories and other scientific establishments. But even after Crosse was pressed into providing details, the experiment did not immediately attract further research. The reason is clear. During the controversy, Crosse never refined his results, nor did he take certain steps (such as excluding the outside air) that critics pointed to as prerequisites to any claim concerning spontaneous generation. Crosse's diffidence (he said he was unable to abandon his research on artificial crystals) defused suspicions that he was sensation-mongering, but rendered any further programme of research by others highly problematical.

As it happened, confirmation came immediately and from the highest authority possible. The newspapers announced at the end of February 1837 that Michael Faraday had successfully repeated Crosse's experiment. Although Faraday had not even attempted to do this, blanket coverage of the story in the press meant that this 'result' joined the original report of Crosse's work as a fact, so that the world's leading experimenter now stood behind the electrical origin of life. By the

• This article is a shortened version of James Secord's chapter "Extraordinary Experiment" in *The Uses of Experiment: Studies in the Natural Sciences* (eds David Gooding, Trevor Pinch and Simon Schaffer). The book was published by Cambridge University Press last year.

end of the year the account had been included in standard works such as the *Annual Register*. Faraday did his best to counteract these reports, but to little avail, and he was invoked in this connection as recently as 1979.

Dislike of popular misapprehension was not the only reason why Faraday tried to disassociate himself from the mites. He feared that his own interest in the convertibility of the 'powers' of nature — electricity, magnetism, gravity, heat and so forth — would all too easily be seen as investigation into the 'power' of life itself. Among all the disciplinary boundaries established around 1800, none was stronger or more significant than the line between matter and life, between the physical and biological sciences. Faraday's attitude was widely shared, and some practitioners had repeated Crosse's experiments simply to show that nothing happened.

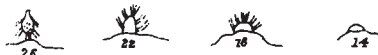
The less *elitist* Electrical Society of London, with a populist, middle-class basis, had been founded in 1837 partly to encourage a more inclusive notion of its subject. By welcoming the acari into its proceedings, it ensured that Crosse's work was not limited to the ephemeral context of popular magazines. Similarly, many medical men remained sympathetic to the spontaneous generation of complex organisms, largely because of the problem of the origin of gut parasites.

At the end of 1837 the mites were firmly in the public realm. But, in general, Crosse had left not only his solutions but his science open to contamination. He had violated some of the most fundamental boundaries of the new specialist disciplines: in a world that drew a sharp limit between the sciences of life and matter, Crosse talked about crystallization and organic materials without distinction. And in a world that demanded specific protocols for experiments on spontaneous generation, he spoke of the all-pervasive nature of electricity and refused to intervene more directly. For this reason, together with fears about the consequences for morals, religion and the social order, the electrical acari had failed to find any part in science. The controversy seemed destined for oblivion.

That it was not forgotten is due to a surgeon and popular lecturer, William Henry Weekes of Sandwich in Kent. Weekes was, as one contemporary put it, "the greatest dealer in [experiments on] thunder and lightning" in Britain save Crosse himself. Weekes commenced work in 1840, aiming to vindicate Crosse from charges of atheism by meeting the criticisms of the original experiments. His most important step was placing the apparatus in a bell jar over mercury. The experiment took over a year to show any results. Then, on 25 November 1841, Weekes noticed that "five perfect insects"

had appeared inside the jar amid a cluster of pyramidal quartz crystals. The critics had at last been countered. Closing off the outside air was not the only way Weekes avoided contaminating his experiments. He ensured that his finding was announced at the Electrical Society before a notice of the meeting was sent to *The Times*, which eagerly ran this fresh chapter in the sensational story. Once again, news of the acari made its way around the world.

During the 1840s and 1850s, the experiments of Weekes and Crosse came into



Stages in the emergence of insects from Crosse's first experiment (running from right to left; the numbers are days of 'gestation').

their own in the great debates about natural law and evolution. The central work here was the anonymous best-seller *Vestiges of the Natural History of Creation* of 1844, written by Robert Chambers, a popular author and publisher in Edinburgh. *Vestiges* ranged from the formation of the Universe, through the records of geology, to the final destiny of the human race. The acari had a critical role to play in showing that a miracle need not be invoked to explain the origin of life.

For many people, this was atheism. The Reverend Adam Sedgwick, professor of geology at Cambridge, advised Crosse "not to meddle again with animal creations; and without delay to take a crow-bar and break to atoms his obstetrico-galvanic apparatus". As a consequence of their discoveries, Crosse and Weekes received anonymous threats of violence. The 'persecution' of Crosse became notorious. The case was compared with classic stories of the oppression of science, such as Galileo's treatment by the Inquisition. Farmers blamed his insects for a blight on the wheat crop, an early example of the kind of fears that have re-emerged with the rise of genetic engineering. An exorcism may even have been carried out by a local priest on the hills above Crosse's country house. Crosse was seen as a lost soul wandering beyond the proper limits of knowledge.

Weekes's experiments gave the acari a new lease of life not only in controversies about science and religion, but also in scientific practice. Henry Noad announced in the 1844 that three experiments of his own had been in progress for 16 months. Alfred Smee, another prominent figure in Victorian electricity, hoped to reproduce Weekes's triumph as part of his effort to establish a discipline of 'electrobiolgy'. Smee and Noad did not obtain positive results, but remained hopeful.

That other researchers attempted replications after Weekes's success is a crucial

point. Had they found insects too, electrical life would have made its way into standard works, and become tied to a body of related results. But this did not happen. No one else — except for Crosse himself — repeated Weekes's work successfully. This was not because the experiments were necessarily flawed, but because there was nothing further one could do with them. In the twentieth century the only experimenters hoping to try the work of Crosse and Weekes have been school children in the United States, led by books such as Frank Edwards's *Stranger than Science* (1959) to believe that Faraday had repeated the experiments successfully. The Royal Institution was deluged with their enquiries during the early 1960s — after all, the creation of life was a superb project for a science fair.

Although Victorian science was specialized, it still tended to be constructed from elements of the surrounding culture. Geologists, medical men, electricians and naturalists thus held a diverse range of attitudes towards the established religious and political order. With scientists engaged in these wider disputes, their right to legislate popular conceptions of the natural world was constantly challenged.

Chambers, Dickens, Carlyle and other authors questioned the authority of science because of its inaccessibility to the public. Just as important, though, was the phenomenon that a journalist like Chambers represented: the emergence of a mass culture of publishing during the early phases of the Industrial Revolution. As a result, scientists confronted audiences of unparalleled diversity and size. Popular images of science could sometimes be controlled, but contact was usually mediated — as it is today — by journalists, editors and publishers.

In the seventeenth century, experiment before a competent audience had been established as a means for creating consensus within science. Its authority depended on like-minded gentlemen agreeing about what they had seen. The circumstances of the early nineteenth century were radically different. Experiment had yet to prove its ascendancy over new printing technologies and a heterogeneous reading public. During the present century channels of communication and the size of the audience have multiplied further, so that tensions between the power of experiment and the community at large have become still more acute. The period that witnessed Crosse's 'extraordinary experiment' holds the roots of one of the most fundamental problems in the relations between popular culture and the culture of science. □

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The greenhouse question (cont'd)

Last week's promise of occasional special contributions to the continuing discussion of the prospect of global warming requires elaboration. Social and economic issues are as important as the strictly technical.

THE first thing to say about the artificially induced global warming is that, if there is a need to interfere with the greenhouse effect, the consequences and, notably, the cost will be enormous. The world's annual consumption of fossil fuel probably at present releases to the atmosphere 25×10^{12} kg of CO_2 . There is no other commodity in world commerce routinely handled on such a scale. World steel production, for example, is less than two orders of magnitude smaller, which is not surprising given that it requires some tonnes of fossil fuel, or its energy equivalent, to produce a tonne of steel. Cereal crops — rice, wheat and corn (maize) — yield a larger amount of grain, probably a little more than 10^{12} kg a year.

The economic value of the CO_2 business is enormous. With crude petroleum at about \$100 a tonne (10^3 kg), the raw materials of the CO_2 production industry may be valued at about $\$2.5 \times 10^{12}$ a year, more than twice the annual federal budget of the United States. Refining costs (conversion into electricity, or the manufacture of gasoline for road vehicles) probably account for a comparable but larger sum. There is no question that the hard core of the CO_2 business is the largest single production process in the world.

Nobody can tell how quickly this business is at present growing, because aggregate production figures are compiled only with a delay of five years or so. But there is every likelihood that CO_2 production is now increasing by about 3 per cent a year. Measures to restrict emissions, even to present rates, are bound therefore to be expensive. Fuel and refining costs forgone might amount to $\$300 \times 10^9$ a year. Substitution of these by other sources, nuclear power or windmills, for example, would be more expensive, perhaps by a factor of three, but would require initial capital investments at least ten times as great. At least in principle, these costs would arise annually.

Those who argue that restrictions should be postponed, or that adaptation to climatic change may be more prudent than avoidance, have at least a good instinct for the worth of dollar bills. Restrictions on CO_2 emission would be severely deflationary, diverting resources from the production of consumer goods and services into the means by which those goods and services are provided. One of the cruel ironies in the threat of global warming is that the costs of adaptation to

climatic change are not nearly as evident, but would also have deflationary consequences.

The weak link in the arguments now rife about the best response to the threat of artificially induced global warming is not that the computer climate models are still shot through with uncertainties, great though they may be, but that there is a much greater uncertainty about the economic consequences of greenhouse-gas restrictions or, alternatively, of adaptation — the costs of wringing extra irrigation-water from apparently arid land, for example.

Plainly it would be a matter of great importance if the economic consequences of restriction (or adaptation) in industrialized countries were so great that foreign assistance programmes were curtailed. For it is very difficult to conceive of any general agreement between sovereign states on the response to global warming that fails to compensate the poor countries of the world for the restriction of practices they have not begun, which argues that foreign assistance programmes should be increased. It will be even harder to persuade those who calculate that they may temporarily benefit from global warming voluntarily, and without some transfer of resources, to forgo them in the interests of others.

The right balance between restriction and adaptation, which must shift with the passage of time, is another imponderable. It cannot be determined without a better understanding of how the world's economy functions. But how will it be possible to optimize the mechanisms of restriction or the incentives for adaptation without falling into the trap of believing that the global economy can be as fully understood as the functionaries of *Gosplan* in Moscow have been pretending of the Soviet economy these past seventy years?

Other questions, still largely open, are a blend of the technical and social that will be unfamiliar to politicians. There is, for example, the question of how to balance against each other the potentially damaging effects of the three principal groups of greenhouse gases — CO_2 , CFCs and other trace gases, of which methane is the most significant. Is what matters the instantaneous concentration in the atmosphere, or the concentration averaged, with some appropriate weighting, over their expected lifetime in general circula-

tion?

This question has been usefully tackled, if in a preliminary fashion, by D.A. Lashof and D.R. Ahuja (*Nature* **344**, 529; 1990). Molecule for molecule, materials such as CFC-12 are more damaging than CO_2 by a factor of about 10^4 , but exactly what that factor is taken to be depends on the relative weight given to present and future warming (which is how politicians will eventually be required to express their concern for future generations) as well as on continuing uncertainty about the lifetime in the atmosphere of the chief greenhouse gas, CO_2 itself.

Other uncertainties are more exclusively technical, but have a potentially important bearing on policy, especially in relation to adaptation. So much should be clear from the important article by Richard M. Adams *et al.* (*Nature* **345**, 219; 1990), which showed that the adverse effects of global warming on US agriculture would be outweighed by increased photosynthesis in the presence of increased concentrations of CO_2 , at least if there were sufficient irrigation water for use in newly semi-arid regions of the United States.

With the best will in the world, the quality of the information on which the estimates of improved photosynthesis (taken as an increase of 35 per cent for soyabeans following a doubling of CO_2 concentration) is based is relatively less secure than, say, the computer predictions of climate, uncertain as they may be.

This is not for want of attention, but because of the notorious difficulty of telling how well laboratory experiments can be translated to the field (in the literal sense).

If the threat of global warming is substantial, numbers derived from studies such as these will be crucial to the determination of good policy. The economic stakes are so high that poor numbers will yield policies which are not merely painful but in themselves calamitous. One of the objectives of the attempt that will be made over the coming months (and years?) to collect together information bearing on greenhouse questions is to encourage a more rounded discussion of an important problem, which is social and political as well as technical in character — and whose international character implies that national declarations of self-restraint may be as ineffectual as unilateral disarmament.

John Maddox

Which way to fusion ignition?

R.S. Pease

If our knowledge of thermochemistry were limited to the results of test-tube experiments, the performance of high explosives and non-reactive gas dynamics, it would be difficult to predict that a match would ignite when rubbed against the strike pad and then burn steadily. A similar problem faces the nuclear-fusion researcher trying to predict what apparatus to build to achieve fusion ignition and steady self-sustained thermonuclear fusion reactions in a high-temperature plasma contained by magnetic fields. In both cases, the complexity of the process dictates that direct demonstration is required to establish the reality of ignition and burn. Fusion ignition is the essential component of the next stage in magnetic fusion research. Experiments to meet this need have been proposed recently¹⁻⁴. A new proposal comes from the University of Texas⁵; and the European proposal, Next European Torus (NET), and the international proposal, International Tokamak Experimental Reactor (ITER), and their competitors have been discussed by Mitchell and Spears of the NET team⁶. Deciding which are the best options is now becoming important.

Fusion ignition occurs when the power deposited in the plasma by thermonuclear fusion reactions alone sustains or increases the thermal content of the plasma against the power losses arising from imperfect thermal insulation and radiation. To achieve a steady burn the subsequent evolution of the plasma magnetic-field configuration, including refuelling and helium ash disposal, must not lead to violent or self-extinguishing instabilities. Steady burn conditions are needed for most envisaged power-producing fusion plants.

Fusion power is deposited in the plasma by the energetic charged fusion products — notably the ⁴He nucleus. The highest such power achieved in present experiments is about 100 kilowatts in the Joint European Torus (JET); it is provided by the charged products of the fusion reaction D(³He, p)⁴He (ref. 6). The plasma temperature is maintained by 13 megawatts of ion-cyclotron resonance heating. If the higher-cross-section reaction between deuterium and tritium were used, and the thermal insulation and impurity content of the high-temperature plasma matched the best conditions so far achieved in JET, then the power deposited in the plasma by α -particles would be in the megawatt range. But it would be only 1/5 to 1/10 of the external heating power. Thus, it is not likely that ignition will be achieved in the deuterium-tritium experiments in JET planned for 1992 or later, although they may not fall far short.

What is needed is an improvement by about a factor of ten in the thermal insulation achieved in JET for a given plasma pressure. Because the physics underlying the thermal insulation is not clearly established, on one hand it is not possible to rule out further improvements in JET itself, and on the other hand uncertainty exists about what margin of safety to design into entirely new apparatus. It is generally agreed that only the tokamak system may achieve fusion ignition in the foreseeable future. The tokamak confines plasma in a toroidal chamber equipped with strong magnetic fields. Its confinement properties depend mainly on the size of the toroidal electric current that flows through the plasma, and which with its self-magnetic field confines the plasma and provides the thermal insulation — the higher the current, the better. In the operating temperature range of 100–300 million K, the ratio of thermonuclear fusion power output to heating power needed to sustain the temperature is approximately proportional to the square of the current, using the simplest empirical scaling law for the thermal insulation available to us⁷. This ratio needs to be improved by about ten times, so that, following the scaling law, an increase in current by about a factor of three over that used in JET could be sufficient.

The table summarizes the present set of proposals for ignition experiments. They are mainly of two kinds. First, a group of three — Ignitor, Compact Ignition Tokamak (CIT) and Ignitex — are all aimed solely at ignition, and aim to achieve it as cheaply as possible, using cryogenically cooled copper coils, small

dimensions and high magnetic fields, with no radiation screening of the coils. The magnetic coils are fully exposed to the flux of neutrons with energies of 14 million electron volts from the deuterium-tritium reactions and so the electrical insulation has to survive high radiation doses, and remote handling will be essential after the first few ignited pulses. The currents are around the minimum estimated value of 10–12 million amps for ignition (although in fact more elaborate scaling laws were used). The dimensions are determined by the maximum magnetic field strength to be used, which, in turn, is determined by the mechanical strength of the copper coils and their supports, and by the desired operational life. The more generous dimensions of CIT allow longer pulse durations and more access for additional heating and plasma exhaust. The duration of the current pulse is set by the rate of temperature rise in the coils. The coils are cooled between pulses by liquid nitrogen and/or helium to maximize this rise time. The limitation of this first group is due chiefly to the short current pulse: it is comparable to or less than the burn time of a charge of fuel, so that the long sustained burn needed in reactor design could not be demonstrated directly. For CIT, the longer pulse duration is expected to allow tests of thermal stability and of the effects of helium accumulation.

The second group, characterized by NET and ITER, a Euratom-US-Soviet-Japan collaborative design, goes for a high current to have a better chance of ignition, and uses shielded, superconducting windings that can sustain long pulses of quasi-d.c. operation. These machines have lower magnetic field strengths and larger dimensions to accommodate the neutron screening between the plasma and the superconducting coils. Remote handling is mandatory for hardware maintenance

TABLE — Tokamaks proposed for ignition experiments

Proposal	Origin	Current (MA)	Major/Minor radius (m)	Stabilizing field (T)	Pulse duration (s)	Hoped-for fusion power (MW(th))
Ignitor	ENEA/Euratom	12	1.2/0.45	13.2	5†	150
Ignitex	University of Texas	12/10	1.5/0.47	20	5†	150
CIT	Princeton/Massachusetts Inst. of Technology	11	2.14/0.66	10	7†	100–500
NET	Euratom	25	6.3/2.05	6	>1,000	1,000
ITER	Int. Atomic Energy Agency	22	6.0/2.15	4.9	>1,000	1,080
JIT	Ref. 4	30	7.5/3	4.5	1,000–4,000	500–3,000
JET*	Euratom	4–7	3.0/1.3	3.4	~10	~10

* JET, not an ignition experiment, was first operated in June 1983. The experiments reported in ref. 6 give results for currents up to 4 million amps. JET is capable of up to 7 million amps in some plasma configurations. Operating at higher current has not yet been fully explored.

† For the inertially cooled systems, longer periods of constant conditions can be obtained by operating at lower magnetic field strengths.

ENEA = Comitato Nazionale per la ricerca e lo sviluppo dell'Energia Nucleare e delle Energie Alternative, Italy.

within the coils. The technology is similar to that envisaged for electricity-producing fusion plants. They have strong additional heating, current drive and complete plasma exhaust systems. They are more expensive than the first group, but will produce a great deal more information. Mitchell and Spears¹ suggest direct typical capital costs of 3–4 thousand million dollars for NET or ITER, and 5–7 hundred million dollars for the first group.

There are also intermediate proposals. The most clearcut is JET, which is a tokamak 'thermonuclear furnace', using radiation-screened and continuously water-cooled copper coils, giving a pulse duration of about 2,000 s to explore the burn phase. It is the simplest and most straightforward of the devices proposed, has a good margin to secure ignition, and can address a number of key issues such as impurity accumulation and exhaust technology, but does not have the development potential of NET and ITER.

These proposed devices are only in the outline-design stage, with some seeking approval to go to a detailed design stage. Decisions on a successor to JET, the equivalent in Japan (JT-60) and the United States (TFTR, D-III-D) are needed if the thrust of fusion research is to be main-

tained, for these experiments are now nearing the end of their development potential. Nevertheless, it is very cost effective to optimize the performance and understanding of JET during the 1990s. One of these proposals may be the successor; small-scale apparatus will not be sufficient. The Ignitor-CIT group may seem to produce only rather small improvements on the current and duration already available in JET; the larger-scale NET and ITER type device will be needed if a net electricity-generating plant is to be built; JET has the best margins and simplest technology. In a worldwide international programme of the present scale, it should be possible to mount more than one type of these new experiments. The knowledge and risks will thereby be spread and, hopefully, the neo-Promethean goal of fusion ignition may be achieved by the turn of the century. □

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POTASSIUM CHANNELS

Mixing and matching

Richard W. Aldrich

THE potassium channels found in excitable and other cell types exhibit a bewildering — and intriguing — diversity. Such channels have been classified according to their biophysical and pharmacological characteristics. More recently, the explosion of published sequences of K⁺ channel proteins has added a molecular dimension, with sequence similarity becoming a criterion for relatedness. Now a new level of complexity in K⁺ channel structure and diversity has been found. As reported by Ruppersburg *et al.*¹ and Isacoff *et al.*² on pages 535 and 530 of this issue, and by Christie *et al.* in *Neuron*³, K⁺ channel components encoded by separate genes can form heteromultimeric channels with properties that are different from those of homomultimeric channels consisting of several copies of an identical subunit.

In *Drosophila* and in mammals, a large number of distinct K⁺ channels have been identified by complementary DNA cloning techniques. In *Drosophila*, many channel variants arise from alternative splicing of the *Shaker* gene transcripts, and from additional genes. In mammals, several genes homologous to *Shaker* have been found, but their transcripts do not undergo extensive alternative splicing. It is widely thought that the large number of genetic variants in part underlies the func-

tional diversity of K⁺ channels. That messenger RNA from any one of the given variants produces functional channels in oocytes has led to the generally accepted idea that a functional K⁺ channel in oocytes is a multimer of identical subunits. The idea that these K⁺ channels function as multimers and not as monomers was originally based on analogy with Na⁺ and Ca²⁺ channels, whose major subunits consist of four internally homologous domains, each of which is similar to a single K⁺ channel subunit.

More direct evidence for multimer formation comes from genetic studies in *Drosophila*. Certain mutant alleles of the *Shaker* gene do not contribute independently when combined in heterozygotes, suggesting that the two mutant gene products interact^{4,5}, for example by aggregating to form multimeric channels. Further evidence has been provided by experiments on transgenic flies transformed with a truncated *Shaker* gene product that does not produce functional channels in oocytes⁶. Flies transformed with the truncated genes have hyperexcitable neuromuscular junctions, a phenotype of *Shaker* mutants that results from decreased levels of K⁺ channels in the presynaptic terminal. This result can be easily explained if the truncated nonfunc-

tional proteins aggregate with the wild-type *Shaker* proteins. Isacoff *et al.*² have carried out a similar experiment with oocytes. They showed that co-injection of mRNAs for truncated and wild-type proteins from *Shaker* yielded much less K⁺ current than injection of wild-type mRNA alone. And evidence from mRNA fractionation experiments, in which co-injection of low and high-molecular-weight fractions into oocytes yields K⁺ channels with properties different from those obtained with only high-molecular-weight fractions⁷, suggests the existence of low-molecular-weight subunits.

The three new reports address the same question: "Can different K⁺ channel proteins aggregate to form heteromultimeric channels with distinct properties?". The basic approach is to express two K⁺ channel variants with different properties in a single cell type (*Xenopus* oocytes or HeLa cells) and assay for properties different from those predicted from independent expression of the two channel types. All three groups used tetraethylammonium (TEA)-binding as a convenient assay, and the K⁺ channels described differ by two to three orders of magnitude in their sensitivity to blocking by TEA. In the different laboratories, the following pairs of channels, one with high and one with low sensitivity, have been coexpressed: RBK1 and RBK2 (ref. 3); RBK1 and RGK5 (ref. 3); RCK1 and RCK4 (ref. 1) (all of these are rat channel variants; RBK1 and RCK1 are identical); and ShB and RCK1 (ref. 2) (*Drosophila* and rat). In each case, a K⁺ current component was identified that had intermediate TEA sensitivity in macroscopic voltage-clamp records. A quantitative analysis of the RBK1–RBK2 and RBK1–RGK5 combinations showed that the resulting TEA-inhibition curves differed from the linear combination of inhibition curves of the two types individually, in any combination³. RCK1–RCK4 combinations also showed intermediate sensitivity to dendrotoxin, a K⁺-channel-blocking toxin¹.

Ruppersburg *et al.* and Isacoff *et al.* also examined voltage-dependent gating properties. RCK1–RCK4 combinations produced a transient current, with intermediate TEA sensitivity, that inactivated with kinetics intermediate between RCK1 and RCK4 and recovered from inactivation faster than RCK4 (ref. 1). ShA–ShB combinations, or tandem dimers made by splicing together cDNAs for ShA and ShB, also gave inactivation kinetics intermediate between ShA and ShB alone². Combinations of ShA and a mutant variant of ShB that has very slow inactivation gave similar results. The inactivation kinetics of the RCK1–RCK4 (ref. 1) and of the ShA–ShB mutant² combinations were found in membrane patches containing only single channels, confirming that the intermediate properties resided in

individual channel molecules. Although inactivation of RCK1–RCK4 was more like that of RCK4, the single-channel conductance was similar to RCK1.

Taken together, these results provide strong evidence for the formation of heteromultimeric channels in heterologous expression systems. They imply that all the possible combinations contribute to the wide diversity of K^+ channels, and thereby raise some interesting questions.

Do heteromultimeric channels form *in vivo*? So far there is little evidence one way or the other. RNA blot hybridization and immunocytochemical staining experiments have revealed different but overlapping tissue distributions of different variants in mammalian brain and in the nervous system of *Drosophila*, suggesting that the K^+ channels in different cells may be composed of different subunits^{8,9}. A definitive answer to this question awaits purification of the channel proteins and determination of their subunit composition by biochemical approaches. If heteromultimers do form *in vivo*, what is the functional importance of the different combinations and the mechanisms controlling differential expression and assembly of the subunits into multimeric channels? What are the structural limits to the ability to form heteromultimeric channels? It is important to note that all of the combinations studied have belonged to a fairly closely related family of K^+ channel components. Several other genes encoding K^+ channel components have been found in flies¹⁰ and in mammals^{11,12} that are more distantly related. Can these components combine with those studied here, or will they combine only with their closer relatives? If cross-family heteromultimers form, will they produce channels that are qualitatively different from the individual homomultimeric channels, and perhaps account for some of the many K^+ channel types that have not yet been isolated using molecular techniques?

As usual, many fresh problems have emerged from the present results. But such is the speed of progress in the study of the molecular properties of K^+ channels that it will not be long before we have at least some answers — and no doubt, a host of further questions. □

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Getting to the core

John Wahr

THE boundary between the Earth's fluid core and surrounding solid mantle is an extraordinarily interesting place. It is the meeting place of two vastly different materials — one a liquid iron alloy of some sort, the other a solid silicate that is possibly at a much lower temperature than the underlying liquid. It is not at all obvious how two such different materials interact. These interactions — mechanical, chemical or thermal — may have important implications for mantle convection, the process responsible for continental drift (for example, heat flowing from the core may drive convection), and for the core dynamo, the self-sustaining fluid flow in the core that generates the Earth's magnetic field. A new paper by Jault and Le Mouél provides an appealing view of the way that one of these interactions may operate.

Information about core–mantle interactions can come from a wide variety of sources, including (perhaps surprisingly) changes in the Earth's rotation period inferred from astronomical observations over the past two centuries. The Earth's rotation period (nominally one day) fluctuates irregularly by up to 4–5 milliseconds over periods of a few decades or so, which has long been recognized as being due to the exchange of angular momentum between the core and mantle: as fluid motions in the core fluctuate, the corresponding changes in the core's total angular momentum must be accompanied by an equal but opposite change in the angular momentum of the mantle.

What is less clear is the mechanism by which angular momentum is exchanged. For many years, most attention was centred on electromagnetic coupling: the interaction, through the Lorentz force, between the conducting mantle and the magnetic field generated in the core. But in 1969, Hide² proposed a different mechanism involving topographic coupling. In this model, fluid flowing horizontally at the top of the core pushes against any topographic features sticking down into the core from the mantle. Averaged over the entire core–mantle boundary, the result is a torque on the mantle from the core (and vice versa). The torque changes as patterns of flow change.

Hide's idea languished for some time, because numerical estimates for the topographic torque were not really feasible until recently. Such estimates require knowledge of the boundary topography and of the horizontal variations in fluid pressure at the top of the core. There are now long-wavelength, global models for both — pressure models are derived from measurements of the magnetic field at the

Earth's surface, and topography models are based either directly or indirectly on seismic data.

But the accuracy of these models is open to question, because the data can be very noisy (particularly those from seismic experiments) and they do not cover the Earth's surface evenly. Furthermore, simplifying assumptions often have to be made. For example, to obtain a fluid pressure model it is useful to assume that the Coriolis and pressure forces at the top of the core essentially balance each other, and that the mantle is a perfect insulator.

The topographic torque estimated by several groups using these models of pressure and topography gives results that are larger, by factors of anywhere from 5 to more than 100, than the measured length-of-day variations. This implies that topographic coupling could be an important source of rotation fluctuations, but also suggests that either the fluid pressure or the boundary topography (or both) are overestimated in the models.

The results of Jault and Le Mouél, however, run counter to that last conclusion. They find that by modifying slightly the topography model — modifications that are comfortably within the error bars of the model and that tend to leave the overall topographic amplitude unchanged — the inferred torque can drop from a value that is two orders of magnitude too large to a value that agrees well with the rotation data. The total torque is sensitive to the location of the topographic features relative to the distribution of fluid pressure. If a protruding bump is placed exactly at the centre of a high-pressure point, the pressure against the sides of the bump will cancel, giving no net torque; but if the bump is slightly off-centre, there will be a pressure imbalance and thus a non-zero torque.

One implication is that, for a given pressure model, just about any topographic model (irrespective of its amplitude) need be altered only slightly to give the correct torque. (By the same token, it probably follows that, for a given topographic model, any pressure model can be similarly adjusted — but Jault and Le Mouél do not consider that case.) For example, by a slight reorientation of a certain topographic pattern, the total eastward torque can be made to nearly cancel the total westward torque, giving a small total torque as seems to be required by the rotation data. Thus until the models are much more reliable than they are at present, the rotation data probably cannot be used to discriminate between them.

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But does it not seem an unlikely coincidence that the large eastward and westward torques should so nearly cancel? Jault and Le Mouel argue to the contrary that this is exactly what one would expect. If these torques on the mantle did not nearly cancel, there would be a large, opposing torque on the core, causing the core and its associated pressure field to rotate with respect to the mantle in the direction of decreasing torque. Thus the core and mantle would oscillate with respect to each other about an equilibrium position of zero torque. Damping of the motion (for example, by electromagnetic coupling) would tend to leave the system

always close to the equilibrium position, executing small oscillations. Jault and Le Mouel even derive a crude estimate of about 60 years for the natural period of these oscillations, which is indeed of the same order of magnitude as that of the observed fluctuations in rotation. □

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VISION

Does the eye grow into focus?

S. J. Judge

If the eye is too long for its optical power, it will be short-sighted (myopic). In the human eye, an excess of 0.2 mm, or about 1 per cent of eye length, is enough to create significant myopia. Yet most eyes are neither short- nor long-sighted — that is, the eye has grown so that the retina lies just where the optical components of the relaxed eye focus the image of a distant object. How does such matched growth come about, and why does it sometimes fail? As became clear at a recent meeting*, there is now good evidence from experimental work that the visual image formed by the eye plays an important part in regulating the eye's growth, and that if the retina is deprived of a visual image, the eye overgrows so that it becomes myopic.

The most extensive studies have been carried out in the chick. Covering one eye with a small translucent hemisphere, thereby making the retinal image essentially uniform and featureless, causes the vitreous chamber — the rear part of the eye between the lens and the retina — to enlarge and the eye to become myopic. The cause is the loss of patterned illumination, not the slight decrease in brightness, or other simple physical factors such as alterations of temperature or intraocular pressure. Could the eye be attempting to look at the hemisphere covering it, and the effort to focus on such a close object be the relevant factor? It seems not, because deprivation myopia still develops when the ability to alter focus is abolished (D. Troilo, University of Oxford).

*Ciba Foundation Symposium No. 155: *Myopia and the control of eye growth*, London, March 20–22, 1990. Proceedings to be published in November 1990 by Wiley, Chichester.

One of the most remarkable findings¹ is that if sectors are cut out of the translucent hemispheres so that the chicks can see in one direction and not another, the part of the eye that is deprived of patterned visual stimulation overgrows, producing a short-sightedness that is specific to that part of the eye (Fig. 1). The existence of such



FIG. 2 Chick with spectacles — eye growth alters to compensate, at least to some degree, for the added lenses. (From ref. 7.)

local effects implies that regulation of eye growth might be accomplished by mechanisms within the eye itself; and the demonstration by Troilo *et al.*² that local myopia still develops after section of the optic nerve has deprived the brain of all information about the retinal image in that eye, reinforces that conclusion.

Increased growth, rather than simply stretch, does indeed occur; DNA, protein and proteoglycan synthesis are increased in the sclera (the eye's rigid outer coat) of deprived eyes^{3–5}. The chain of events

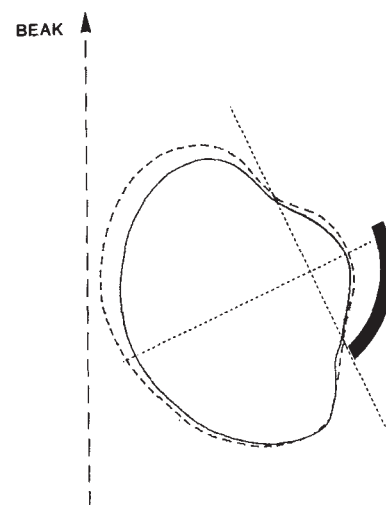


FIG. 1 When the side and rear field of view of one eye of a chick (seen from above) is blocked out with a piece of translucent plastic (solid arc), the corresponding part of the eye overgrows (dashed line), making that part of the eye selectively short-sighted relative to a normal eye (solid line). (From ref. 2.)

linking visual stimulation and ocular growth is unknown, although myopia still develops if the retinal ganglion cells — the output cells of the retina that send signals to the brain — have been killed (C. F. Wildsoet, University of Queensland). It is suspected that one class of retinal neurons, the amacrine cells, is involved in regulating growth. Dopamine, a chemical transmitter by which some retinal cells signal to others, is implicated in that stimulating dopamine receptors can prevent deprivation myopia developing, in both the chick and the monkey (R. A. Stone, University of Pennsylvania).

One area of complete ignorance is how any putative factor might travel from the retina to the sclera — particularly how the signal first crosses the barrier of the retinal pigment epithelium, and is then somehow constrained from becoming widely distributed in the blood vessels of the choroid.

Whatever the mediatory mechanism is, the observation that periods of normal vision as short as two hours a day prevent myopia developing (J. Wallman, City University of New York) is an intriguing one. It rules out the idea that the relevant signal might be some simple function of average retinal image contrast, and may relate to the fact that a true regulatory mechanism has to avoid responding to the blur created on large parts of the retina when animals focus on close objects, as chicks do when feeding.

But the most important issue is whether or not the effects that have been observed

with deprivation are produced by a mechanism that normally regulates eye growth so that the eye grows into focus. A good control mechanism should be able to modulate growth up and down. It seems that such ability does exist, but whether the mechanism is entirely intraocular is not clear. If deprivation myopia is induced in the chick eye and then normal vision is restored (at a time in life when the eye is still growing) the eye compensates by slowing growth of the vitreous chamber⁶. One of the important new findings presented at the meeting was a demonstration that mammalian eyes can also recover from deprivation myopia (T.T. Norton, University of Alabama). It turns out that rearing in darkness produces a different effect from form deprivation, making chick eyes long-sighted (hyperopic). Chicks made hyperopic by dark-rearing and then brought into the light recover through increased growth of the vitreous chamber. Recovery from either myopia or hyperopia occurs when accommodation has been eliminated; recovery also occurs, though less effectively, when the optic nerve is cut (D. Troilo).

Furthermore, chick eyes alter their power so as to compensate for spectacle lenses⁷ (Fig. 2) or contact lenses (J. G. Sivak, University of Waterloo), and these

effects also persist after accommodation is abolished⁸. One part of the eye can even grow selectively to compensate for local refractive demands such as those presented by low ceilings⁹. For eye growth to adapt to both imposed myopia and hyperopia, a signal related to the sign of the defocus is needed. The key issue is whether this signal can be extracted within the eye itself, from the blurred retinal image. The eye and brain together can discern the sign of defocus because it is used to adjust accommodation. But can the eye itself do so, and if so how? This remains one of the most intriguing questions of eye growth regulation. □

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DARK MATTER

Searching for MACHOs

Bernard Carr and Joel Primack

ONE of the most important problems in cosmology is to identify the dark matter that is thought to reside in galactic halos and clusters of galaxies. In recent years much attention has centred on the possibility that this consists of elementary-particle relics of the early Universe, such as axions or 'Weakly Interacting Massive Particles' (WIMPs). Experiments to detect them as they stream through the Earth are already underway in both the United States and Europe. Another possibility is that the dark matter is present as some sort of astrophysical object. This idea derives from the suggestion that most of the gas in the Universe may have been processed through a generation of 'Population III' stars formed relatively early in the life of the Universe. Dark remnants of these stars have been termed 'Massive Compact Halo Objects' (MACHOs) by Kim Griest of Berkeley.

Prominent in the search for WIMPs has been the newly established Center for Particle Astrophysics (CfPA) at Berkeley, California. But because it is still an open question whether the dark matter consists of WIMPs or MACHOs, the Center is considering looking for MACHOs as well.

Recently it hosted a one-day workshop* on the topic, at which there was great enthusiasm for the idea of searching for MACHOs in our Galactic halo using gravitational microlensing.

MACHOs derive from the sort of baryonic matter associated with atomic nuclei, whereas WIMPs are an example of more exotic, non-baryonic matter. The most recent calculations of cosmological nucleosynthesis¹ imply that the average density of baryonic matter in the Universe lies in the range 0.04–0.06 times the critical cosmological density (this density corresponds to a universe that contains just enough matter for gravity to halt its expansion). This range assumes that the Hubble constant, which determines the rate of inflation of the Universe, is 50 km s⁻¹ Mpc⁻¹. Because inflationary models require that the total density be very close to the critical value, this implies that non-baryonic dark matter must exist. On the other hand, the density of visible baryonic matter is just 0.01 times the critical value, so some baryonic matter must also be dark.

The dark baryons may be in the form of hot intergalactic gas (J. Bartlett, Berkeley), in which case its temperature would have to be in the range 10⁴–10⁸ K; but it is also possible that some baryons may have

become MACHOs. This entails several possibilities that depend on whether the non-baryonic matter can cluster inside galactic halos and on whether the MACHOs formed before or at the same time as galaxies (B. Carr, University of London). If the non-baryonic matter does not cluster, halos must consist almost entirely of MACHOs. However, WIMPs are the type of non-baryonic dark matter that would cluster and, in this case, halos would contain both species. If the MACHOs are pregalactic, the ratio of WIMP to MACHO density should be everywhere of order 10; but if MACHOs formed during the first phase of collapse of matter into protogalaxies, they should be confined exclusively to halos and this would decrease the WIMP-to-MACHO ratio because the MACHOs would be more concentrated.

MACHOs can, it seems, take one of two forms². Either they are the remnants of a population of 'Very Massive Objects' (VMOs) — stars larger than 100 solar masses ($M_{\odot}$) — which collapsed to black holes during their oxygen-burning stage, or they are objects that are too small to burn hydrogen at all ('jupiters'). If they are VMO remnants, they would have two important observational signatures: a background of gravitational radiation generated by collapse to the black hole and a background of electromagnetic radiation generated by the VMOs' nuclear-burning phase.

Support for the VMO idea has suffered from the finding by the Cosmic Background Explorer that the microwave background spectrum has an almost perfectly thermal form (A. Lange, Berkeley) — there is no sign of the VMO electromagnetic signature, although it might still be found in the near-infrared³. Most attention at the workshop was therefore directed towards the jupiter hypothesis. Possible, although rather controversial, support for this comes from the claim that cooling flows of gas at the centres of rich clusters of galaxies seem to be creating low-mass objects with high efficiency, perhaps as a result of high pressures⁴. This could not produce dark matter in halos, but smaller-scale cooling flows might have done so at pregalactic or protogalactic epochs. Such cooling flows would tend to produce jupiters in clusters, although the clusters would be broken up by collisions if close enough to the galactic centre.

The most interesting consequence of jupiters would be their gravitational lensing effect. Lensing occurs by the bending of light in a gravitational field, and comes in two varieties. 'Macrolensing' occurs when the light from a distant object, such as a quasar, is bent by the gravity of an intervening galaxy or cluster to produce multiple images. 'Microlensing' occurs when an individual halo object crosses one

*Workshop held at CfPA, Berkeley, California, on 26 March 1990.

of the macrolensed images, thereby causing its brightness to vary relative to the other images. The latter is detectable only for halo objects with masses below $0.1 M_{\odot}$, because the timescale of the fluctuation increases as the square root of the deflector mass. So microlensing can be used to look for jupiters but not for the more massive remnants.

Irwin *et al.*⁵ already claim to have observed quasar microlensing, with the deflector mass in the range 0.001 – $0.1 M_{\odot}$. But the interpretation is complicated by the fact that the light path goes through the centre of the lensing galaxy, which makes the deflector mass uncertain (C. Kochanek, Berkeley). Moreover, near the centre of a galaxy the most likely deflectors consist of ordinary matter rather than MACHOs. It may be more promising to look for microlensing of high-redshift supernovae⁶ because these are compact, abundant and have well understood brightness variation (E. Linder, Stanford).

Another possibility is to seek microlensing by MACHOs in our own Galactic halo⁷ by looking for variations in intensity of stars in the Large Magellanic Cloud (LMC) (K. Griest). In this case the timescale of the intensity variation is shorter, so it would be practical to look for VMO black holes as well as jupiters. But the probability of a particular star being microlensed is only 10^{-6} , so one has to look repeatedly at many stars.

Two groups have begun searches for such local microlensing. A French group (J. Rich, Saclay) is concentrating on analysis of Schmidt telescope plates of the LMC, several hundred of which are available at present. Their preliminary conclusion is that it is possible to carry out reproducible photometry on these plates with sufficient accuracy to search for microlensing. Both they and a group at the Lawrence Livermore Laboratory (C. Alcock) are studying the possibility of repeatedly imaging the LMC with a charge-coupled-device (CCD) camera on a small dedicated telescope in the Southern Hemisphere. The large-amplification events that would provide the most convincing signal should not be uncommon, and because they would last only for a very short length of time, the requirement of frequent sampling favours this approach over the use of plates.

A CfPA/Steward Observatory collaboration (S. Perlmutter) is studying intrinsic stellar variability, as this may mimic

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In the shadow of Vesuvius



MOUNT Vesuvius, near Naples, Italy, provided the generic example of a so-called 'plinian' eruption when it destroyed Pompeii and Herculaneum in AD 79. Plinian eruptions are those of an explosive, magmatic type, characterized by a high debris plume and widely dispersed fallout deposits. On page 519 of this issue, Sigurdsson *et al.* show that the pumice deposits from that historic outburst preserve evidence of mixing processes in the chemically stratified magma chamber during eruption.

The vivid depiction of Vesuvius in the throes of eruption by the English painter Joseph Wright, pictured above, may well be a work of imagination rather than recollection, as there is no record of eruptive behaviour during his visit to Naples in 1774. Nevertheless, the picture is strongly reminiscent of the description of the AD 79 event by the younger Pliny: "Ashes were already falling, hotter and thicker as the ships drew near, followed by bits of pumice and blackened stones, charred and cracked by the flames . . . Meanwhile on Mount Vesuvius broad sheets of fire and leaping flames blazed at several points, their bright glare emphasized by the darkness of night . . . the buildings were now shaking with violent shocks, and seemed to be swaying to and fro as if they were torn from their foundations. Outside on the other hand, there was the danger of falling pumice-stones, even though these were light and porous . . .". Sigurdsson *et al.* find that this pumice changed from white to grey several hours into the main eruptive phase, corresponding to a change in parent magma type from silica-rich to magnesium- and iron-rich.

Philip Ball

microlensing. Because studies of stellar variability in the past have been based on series of plates, little is known about short-term or non-periodic variability. The collaboration is using the database from the Steward Observatory CCD Transit Instrument, which for the past two years has been storing data at a number of wavelengths from stars that pass overhead. The fact that an increase in a star's brightness due to microlensing is independent of wavelength should help to distinguish lensing events from intrinsic stellar variations.

The different MACHO scenarios require different searching strategies. If MACHOs are clustered, the clusters would be optically thin but one would

need to look at large areas of the LMC to find them. If the galaxy halo is a mixture of WIMPs and MACHOs, the MACHOs may be more abundant nearer the centre, in which case a dedicated telescope should ideally search for microlensing towards the Galactic bulge during the seasons in which the LMC is below the horizon. But the important message is that the search for microlensing seems immediately feasible. □

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Tracking an elusive receptor

Regis B. Kelly

AFTER newly synthesized proteins reach the inside of the endoplasmic reticulum (ER), folding and oligomerization are facilitated by resident ER proteins such as heavy chain-binding protein (BiP) and protein disulphide isomerase (PDI). Although these resident ER proteins are soluble, they are selectively retained in the ER while newly synthesized soluble proteins leave on their way to the Golgi complex. Selective retention of the resident ER proteins is thought to involve a highly conserved sequence on their carboxy terminus, KDEL (Lys-Asp-Glu-Leu), which is bound by an integral membrane protein¹, the KDEL receptor. But although KDEL-terminating proteins are abundant in the ER, no ER membrane protein is present in sufficient concentration to bind to all the KDEL-terminating proteins and keep them in the ER. To resolve this quandary, the existence of a post-ER¹ or salvage compartment² was predicted in which KDEL receptors capture KDEL-terminating proteins and return them to the ER. Vaux and colleagues, reporting on page 495 of this issue³, have now experimentally validated this imaginative and, at the time, daring prediction by identifying and localizing the KDEL receptor using an anti-idiotypic strategy.

For an anti-idiotypic strategy to work, antibody binding sites that recognize KDEL-containing peptides must resemble the surface regions of the KDEL receptor to which the same peptides bind.

If such anti-KDEL antibodies are used for immunization, the second generation of antibodies (anti-idiotypic or network antibodies) will include those that recognize the KDEL-binding site on both anti-KDEL antibodies and on KDEL receptors. The new work and other recent successes^{4,5} remind us how powerful an anti-idiotypic strategy can be in revealing protein-protein interactions too weak to be identified in more conventional ways.

The concentration of the major resident proteins in the ER — BiP, PDI, calreticulin and glucose-regulated protein 94 (GRP 94) — is very high, about 400 μM or 100 mg ml^{-1} . A newly synthesized protein encountering the environment in the lumen of the ER is assisted to fold and oligomerize correctly. If folding and oligomerization cannot occur the newly synthesized protein can remain tightly bound to BiP.

The major resident proteins can also bind up to 300 nmol calcium per mg protein, but with a low affinity (half-maximal binding at 3 mM)⁶. The presence of such a large calcium storage capacity is consistent with the suggested role for the ER as a store of intracellular calcium. Alternatively, or in addition, calcium binding may be required for the folding and oligomerization of new proteins.

Because the resident ER proteins do not escape even in transfectants that overproduce BiP¹, the retention mechanism must be very effective. The model for retention proposed by Munro and Pelham¹

postulated a post-ER salvage compartment, a KDEL receptor, and recycling between the salvage compartment and the ER (Fig. 1). The existence of the post-ER salvage compartment is now well established. Antibodies to salvage compartment proteins identify tubules and vesicles clustered around the Golgi complex. When membrane traffic out of the salvage compartment is blocked by lowering the temperature to 16 °C, the salvage compartment becomes more condensed and visible⁶. When the temperature block is removed, components of the salvage compartment spread like a wave through the ER, convincingly demonstrating that they are being recycled. Vaux *et al.* now provide evidence for the existence of the KDEL receptor. According to the model there should be a membrane-associated

protein in the salvage compartment that binds carboxy-terminal sequences ending in KDEL. Conventional approaches to purification of this protein using affinity columns were not encouraging. Vaux *et al.* achieved success by generating anti-idiotypic antibodies using three strategies (Fig. 2), each giving rise to antibodies recognizing the KDEL-binding site on anti-KDEL antibodies and an integral membrane protein of relative molecular mass 72,000 (M_r 72K) present in all vertebrate species tested. To prove that the 72K protein is part of the KDEL receptor, the authors showed that third-generation antibodies raised to the combining-site regions of anti-receptor antibodies could recognize peptides ending in KDEL.

The authentic KDEL receptor has not so far been characterized, but there has been preliminary characterization of a soluble fragment isolated from hybridoma supernatants. The fragment retains the ability to bind PDI (with a dissociation constant of between 20 and 50 μM) when binding is measured at relatively high calcium concentrations. When used to examine the subcellular location of the KDEL receptor, the antibody has the same distribution as the salvage compartment antigens described earlier, and the redistribution of the antigen and the KDEL receptor is the same in response to a 16 °C block. We can conclude that the KDEL receptor, as predicted, recycles between the ER and the salvage compartment.

Implicit in a recycling model is that binding is favoured in one compartment and dissociation favoured in the other. Vaux *et al.* suggest that differences in divalent cation distribution between compartments might switch the KDEL receptor between affinity states because binding is "divalent cation-dependent". Unfortunately the authors do not say which cations they used and whether binding is enhanced or inhibited. From the conditions of their binding assays, however, it seems as if calcium favours binding. If so, a recycling model would require a low concentration of free calcium in the ER and a higher one in the salvage compartment (Fig. 1). Perturbing the cell's calcium metabolism might perturb the retention mechanism just as lysosomotropic drugs disrupt the targeting of lysosomal enzymes. Evidence for such an effect comes from the observation that proteins normally retained in the ER are secreted from mammalian cells treated with calcium ionophores⁷, or from yeast with defective Ca^{2+} pumps⁸. Cobalt, which blocks calcium channels, inhibits the exit of pancreatic peptides from the ER⁹. So one way in which the calcium-binding properties of resident ER proteins might influence folding and oligomerization of proteins is by regulating the free-calcium concentration in the lumen of the ER.

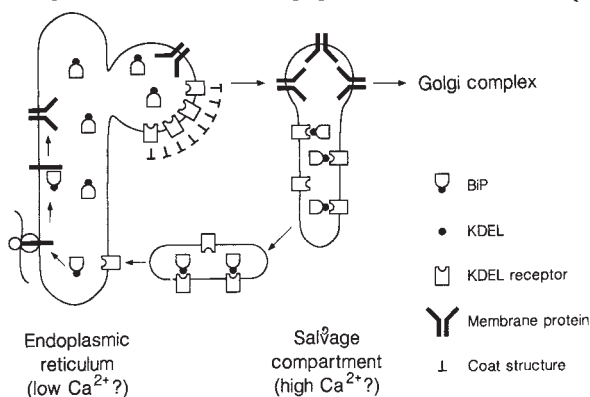


FIG. 1 Speculative model for the retention of ER proteins such as BiP by a recycling mechanism. A newly synthesized membrane protein associates with BiP during or after synthesis until the protein oligomerizes. As the membrane protein leaves the ER some BiP escapes with it. In the salvage compartment the newly synthesized membrane proteins are segregated from the KDEL receptors. KDEL receptors bind the free BiP perhaps because the high calcium concentration in the salvage compartment favours binding. The KDEL receptors carrying the escaped BiP are returned to the ER in a transport vesicle¹². After the vesicle fuses with the ER, BiP dissociates from the KDEL receptors, perhaps because a low free calcium-ion concentration favours dissociation. To allow efficient retention of BiP without vast numbers of KDEL receptors, the empty receptors might be quickly retrieved from the ER by an active process, which could involve a coated structure. Retention of BiP might also involve crosslinking of resident ER proteins into a loose matrix (not shown).

Calcium binding to resident ER proteins might also facilitate retention by crosslinking them into a loose matrix from which they can escape only rarely. GRP94 and calreticulin have polyacidic clusters of amino acids, which are probably sites of low-affinity calcium binding¹⁰. It has been suggested that such acidic regions might be anchors for calcium bridges that would zip together ER proteins, or ER proteins and phospholipids, to form a loose matrix of resident ER proteins. An alternative explanation for the induction of resident-ER-protein secretion by treatment with calcium ionophores⁷ is the disruption of such a calcium-based matrix. Restricted diffusion of resident ER proteins is also implied by the rate of secretion of BiP from which the KDEL sequences have

been removed. The half-time of secretion, about 3 hours, is much slower than would be predicted from unhindered bulk flow.

A calcium-based matrix is a parsimonious explanation for selective retention of resident proteins in the ER. A similar calcium-based model has been proposed to explain another example of retention of proteins from a default pathway, that is the selective condensation of secretory proteins in the *trans*-Golgi network and their packaging into secretory granules^{9,11}.

To explain the remarkably efficient retention of the ER resident proteins by a recycling model, empty KDEL receptors must leave the ER more efficiently than the resident proteins (Fig. 1). If this is the case, blocking the return of membrane from the salvage compartment to the ER by lowering the temperature to 15 °C should cause an accumulation of empty receptors in the salvage compartment. The data of Vaux *et al.* are fully consistent with this prediction. The exit of empty KDEL receptors from the ER could be facilitated by an active-transport mechanism (Fig. 1), analogous to that used for the selective endocytosis of the low-density lipoprotein receptor, for example, from the plasma membrane. If resident ER proteins leaked out with a half-time of 3 hours and an active salvage cycle took only 2 minutes, then only one KDEL receptor would be required for about every 100 KDEL-terminating proteins. Thus restricted diffusion in the ER, together with an active recycling process, could explain the original dilemma — the apparent paucity of KDEL receptors.

A rapid-recycling mechanism might also explain the unusual distributions of salvage-compartment components in cells

treated with brefeldin A (ref. 12). In such cells, *cis* and medial Golgi markers first appear in the salvage compartment but are then transported throughout the ER. The resident components of the salvage compartment, under the same conditions, do not spread widely in the ER, but remain associated with the salvage compartment. The difference between the behaviour of Golgi markers and salvage-compartment markers is most readily explained if both leave the salvage compartment with high efficiency, but only the salvage compartment markers, including the KDEL receptor, are returned by a selective highly efficient transport system.

Although rapid selective transport out of the ER is very attractive on theoretical grounds, experimental evidence for such a pathway is admittedly slim. But given the remarkable success of previous predictions in this field, it is certainly worth looking for. □

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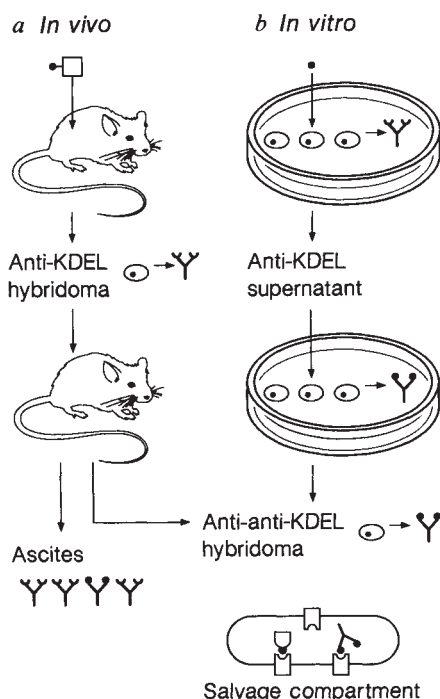


FIG. 2 Antibodies to the KDEL receptor generated by three variations of the anti-idiotypic strategy. *a*, Mouse is immunized with a KDEL peptide that matched the carboxy terminus of the resident ER proteins, but is attached to a carrier protein to facilitate the immune response. Hybridomas that recognize the peptide, anti-KDEL hybridomas, are selected by conventional techniques. When hybridomas are injected into a second syngeneic mouse, antibodies are generated to regions of the anti-KDEL antibodies around the peptide-binding site, the idiotopes. Some of the anti-idiotypic antibodies will bind to the KDEL peptide-binding site on the antibody and also the KDEL-binding site on the KDEL receptor, a 72K protein localized in the salvage compartment. According to the network theory of the immune response, a mouse generating antibodies to an antigen will at the same time generate antibodies to the idiotypic regions of the first antibody. Consistent with this theory, ascites-fluid rich in anti-KDEL antibodies also contains antibodies that bind the KDEL receptor. *b*, As described more fully elsewhere⁴, antibodies to the KDEL peptides are generated by adding the KDEL peptides without carrier to dissociated spleen cells from nonimmunized mice. The culture supernatant containing anti-KDEL antibodies is then used in a second immunization *in vitro* to generate clones of cells expressing anti-idiotypic antibodies. All three approaches yield anti-KDEL-receptor antibodies.

SEISMICITY

Are earthquakes chaotic?

Bruce R. Julian

In 1985, the United States Geological Survey predicted that, before 1993 and probably around January 1988, a magnitude 6 earthquake would occur on the San Andreas fault near Parkfield, California. This prediction was based on the apparent regularity of the seismicity there. Large earthquakes occurred in 1857, 1881, 1901, 1922, 1934 and 1966, and although the sizes and locations of the earlier events are uncertain, there are a startling similarities between the later earthquakes, for which good records exist. The ruptures of the 1966 and 1934 earthquakes, and probably the 1922 one, nucleated in the same place and propagated in the same direction for about the same distance. Moreover, the 1966 and 1934 earthquakes both had magnitude 5 foreshocks located in the same place 17 minutes before the main shocks. But despite this regular be-

haviour, the predicted Parkfield earthquake has not yet materialized. Although nearly three years of the prediction interval remain, many seismologists now question the predictive value of seismicity patterns. In particular, they have begun to wonder whether earthquake occurrence might be an example of the 'deterministic chaos' that many nonlinear dynamical systems can exhibit¹. A paper by Huang and Turcotte² now demonstrates that one of the simplest — although for that reason highly idealized — models imaginable for a fault can indeed exhibit classical chaotic behaviour.

Earthquake occurrence is generally a complicated process — periodicity such as that observed at Parkfield is unusual. Methods for extrapolating recurrence patterns into the future have proved problematic, and the number of suggested

precursory phenomena for large earthquakes (such as quiescence, increased activity and changes in magnitude-frequency statistics) is comparable to the number of investigators. The irregularity of recurrences has usually been attributed to complexity in the underlying mechanism and particularly to the geological heterogeneity of the Earth. This is very different, however, from saying that earthquake occurrence is fundamentally chaotic, which would imply that it is controlled by comparatively simple rules. If earthquakes are truly chaotic, they may actually be easier to predict over short periods of time. On the other hand, because chaotic systems are sensitive to initial conditions in a way that increases exponentially with time, there would be an absolute limit as to how far in the future earthquakes can be predicted, just as there is with weather predictions.

Earthquakes are generated by rapid, unstable slip on faults, which occurs when the frictional force decreases with increasing slip or slip speed. Following Burridge and Knopoff³, many people have modelled faults as systems of blocks connected by springs both to each other and to a 'driver' that moves the blocks slowly along a surface. Although many of these studies have been complicated, involving many blocks, spatial heterogeneity or a variety of physical effects, even simple models can exhibit surprisingly complicated behaviour. Nussbaum and Ruina⁴ considered the case of just two identical coupled blocks linked by identical driving springs and controlled by a simple static/dynamic friction law. When time and distance are scaled, the model is specified by a single parameter: the ratio of the stiffness of the coupling spring to that of the driving springs. The dynamics of the system can be analysed in terms of the relationship between the difference in the elongation of the two driving springs immediately before a 'slip' event and this difference before the previous event. It is found that the behaviour of the system can vary along the length of the 'fault' even though the system is homogeneous. This spatial dependence either may persist — one block always moving in shorter slip events than the other — or may change over time, the shorter events alternating between one block and the other. This suggests that spatial asymmetries observed on real faults, such as the dichotomy between steadily creeping and locked sections, need not be caused by variations in the geological structure or physical state of the rocks, and that the temporal behaviour of a fault could vary qualitatively.

Nevertheless the behaviour of symmetric two-block models with static/dynamic friction is not chaotic: for all coupling ratios and initial states, the motion eventually becomes periodic.

Huang and Turcotte² have bridged the gap between complex and truly chaotic behaviour by introducing asymmetry into the friction coefficients of the two blocks. The two-block system can then exhibit steady sliding, periodic slip events, or chaotic motion. If initially the parameters are symmetric (giving periodic behaviour), and asymmetry in the friction is then introduced gradually, a succession of subharmonic frequencies appears in the blocks' motion, leading to more complicated periodicity and finally to chaos. Such a classical 'period-doubling' cascade is the most common route by which nonlinear systems undergo a transition from periodic to chaotic behaviour. Chaotic behaviour is probably possible also for similar systems containing many blocks or for continuous elastic media. Moreover, if a more realistic friction law with two time-dependent 'state variables' is used, chaotic behaviour is possible with just one sliding block^{5,6}.

Are real, as opposed to model, earthquakes chaotic? This question has been approached only recently. Horowitz⁷ examined a catalogue of 15,196 small earthquakes from Parkfield for the period 1980 to mid-1986, seeking evidence for underlying deterministic behaviour. By arranging all the possibly relevant observed quantities (such as hypocentral coordinates, magnitudes and inter-event times) of the earthquakes into a multi-dimensional sequence, one can apply computer-based methods to test whether the points are distributed in a random way in the 'observation space' or whether they lie in a subspace of lower dimension, suggesting that fewer variables are needed to describe the behaviour. Horowitz constructed a 15-dimensional parameter space for the Parkfield data; the results suggest, albeit ambiguously, that there is an underlying structure with only about six degrees of freedom. So a dynamical system described by six variables should mimic the seismic behaviour of the region. If so simple a system can produce behaviour as complicated as that recorded in earthquake catalogues, it will provide strong evidence that a chaotic process is involved. Whether this will be borne out by future modelling and observational studies remains to be seen. □

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Sensing the truth

THE dilation or contraction of the pupils of our eyes is not merely a device for optimizing the brightness of the image reaching our retinas. It has an emotional component too. Our pupils dilate to study an interesting object or an appealing picture, and contract against an emotionally unpleasant one. This automatic and unconscious response cannot be faked. One group of abstract-art enthusiasts showed strong pupil contraction when exposed to pictures they claimed to appreciate!

Daedalus sees this as a wonderful new technique of market research. His 'collective pupilometer' quantifies the reaction of an audience to presented images. Its digital TV camera identifies the eyes in a face or group of faces by advanced pattern recognition, computes their average pupil area with due correction for ambient brightness, and displays the result.

Capitalist democracy will take another step forward. The advertiser will display prototype posters or commercials to an invited audience under pupilometric surveillance, and pick the people's choice. Film makers will compare the emotional impact of differing scenes, consumer-product manufacturers will rush to test competing masterpieces of pointless plastic styling, and fashion designers will at last have a basis for comparing their eccentric creations. The pornographer will steer his products towards maximum perceived depravity, while the avant-garde artist will measure his exact success at outraging the middle classes.

But Daedalus hopes to extend the principle to another sense entirely, that of hearing. For our ears have a form of 'pupil', too: the stapedius muscle that tightens against excessive noise, reducing the ear's sensitivity like the automatic gain control of a radio receiver. By analogy, this too should have an emotional component — Daedalus recalls those deaf people who mysteriously manage to hear remarks concerning themselves. The flexing of the stapedius muscle should deform the skin above it, 'wagging' the ear slightly. With good fortune, this will be perceptible to an audiologist who knows what to look for; otherwise strain gauges or myographic electrodes may have to be stuck round the ear to detect it. Either way, the subject's emotional reaction to any sound should be immediately apparent.

While Daedalus's 'stapediometer' should be popular with composers and radio producers, and with those who dub the voices of heroes and villains onto film, its main benefits will be psychiatric. With its subtle aid, the scientific psychiatrist will home unerringly onto those topics that most perturb or excite the patient. Traditional, unguided, interminable psychoanalysis will sharpen up and get to the point much sooner.

David Jones

Contamination from satellites

SIR—Radioastronomers must contend with many forms of man-made interference. Although some relief is provided by locating radio observatories in regions remote from industrial activity, our most important protection is the allocation by the International Telecommunication Union of frequency bands where transmissions which could disturb astronomical measurements are restricted. With the proliferation of artificial satellites and the approaching saturation of the electromagnetic spectrum, the preservation of these quiet bands assumes even greater importance.

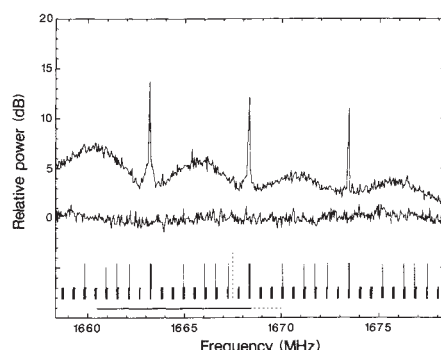
In this context it is disturbing to realize that one particularly important band, the 1660.5–1670.0-MHz band, which contains spectral lines of interstellar and circumstellar OH, is already seriously contaminated with spurious emissions from satellites. Interference was first noticed in May 1989 while observing OH in supernova remnants¹, and a systematic investigation of its origin was begun. It was soon apparent from the temporal behaviour of the signals, from their polarization and from measurements of doppler shift, that the signals originated in satellites. A strong interfering signal at 1667.4900 MHz was subsequently identified with satellites in the US global positioning system (GPS)². Similar signals at 1660.9275, 1661.4900, 1663.2250, 1663.2725, 1664.9600 and 1668.3350 MHz have since been identified as originating in the Soviet Union's GLONASS navigational satellite system³.

Each navigation satellite broadcasts a 'spread spectrum' signal which produces strong sidebands well beyond the allocated frequencies. Dixon has explained the sideband structure⁴. Those narrow sidebands which fall within the radioastronomy bands produce spurious 'spectral lines' with flux densities over 10 kJy (10^{-22} W m⁻² Hz⁻¹). This is roughly a million times stronger than some of the astronomical spectral lines studied with our 26-m radio telescope. The spurious signals are so strong that they are regularly detected in sidelobes of the receiving antenna. In general, interference received in near sidelobes shows up in a single integration period (say 10 min) and can be rejected. A more serious problem concerns spurious signals picked up in far sidelobes which are only apparent after many hours of integration and may then be indistinguishable from spectral features in the astronomical source being studied. I suspect this has already occurred in some of our preliminary studies.

For the GPS system, frequencies of the n th sidebands are $1575.42 \pm 10.23n$ MHz. Only one of these frequencies lies in the 1660.5–1670.0-MHz band, but the

GLONASS system produces many such frequencies. For GLONASS, the frequencies of the n th sidebands are given by $1602. + 0.5625m \pm 5.11n$ MHz, where m takes a different value in the range 1 to 24 for each of 24 different satellites. When this system is complete it will produce 42 separate spurious signals in this radioastronomy band. During February 1990, I have confirmed that five of these satellites generate strong spurious signals in this band. The figure shows the spectrum of one of the GLONASS satellites, obtained by pointing the antenna directly at the satellite while scanning a single 10-kHz-wide channel across the band. As well as the three spikes, two of which are within the radioastronomy band, there are broad humps between the spikes and narrower shoulders close to the spikes. Power in these humps will contaminate continuum and extra-galactic spectral measurements while the spikes contaminate galactic spectral-line measurements.

I have examined ten of the presently active GPS satellites, six of which emit strong spurious emission in the ninth side-band at 1667.49 MHz and four of which do not. The six offending satellites were all launched before 1986. The fact that all GLONASS satellites and most of the GPS satellites emit considerable power in the 'protected' radioastronomy band indicates a lack of appreciation by system designers for the extremely low flux levels of astronomical radio sources



The upper trace is a spectrum of a GLONASS satellite in and near the radioastronomy band showing the 10th, 11th and 12th sidebands resulting from 'spread spectrum' modulation of the central frequency 1612.125 MHz. The lower trace was recorded with the antenna pointed about 10 degrees away from the satellite. Short vertical lines indicate frequencies where similar spikes will occur if the entire 24-satellite system is completed. Longer, vertical lines mark frequencies emitted by satellites which are now transmitting. Most GPS satellites emit a strong spurious signal at the frequency shown by the dashed vertical line. The band 1660.5–1668.4 MHz, where radioastronomy and passive space research share exclusive primary allocation is indicated by a solid horizontal line; the dashed extension indicates a shared band.

and the high sensitivity which a radio telescope achieves through long integration times. On the other hand, the absence of this type of interference from recently launched US satellites indicates that suppressing such spurious emission is technically feasible.

The problem of pollution of radioastronomy bands is already serious⁵. In particular, establishment of the GLONASS system has now seriously curtailed radio studies of important classes of stars⁶. Both the GPS and the GLONASS systems were designed for the same purpose, the precise navigation of military craft, and neither is complete. Both, particularly the GLONASS system, are incompatible with continued observation of OH in astronomical sources. I strongly urge that future systems be compatible with the needs of radioastronomy and that existing systems causing interference be phased out.

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Transmission of leukaemia

SIR—There has been much recent discussion about the risk of leukaemia in children whose fathers had been occupationally exposed to radiation^{1,2}. One crucial point, the possibility of an internal dose burden to the male gonads, has been neglected. The basic requirement for such an event is that relevant radionuclides are retained selectively by testicular tissue.

This phenomenon was investigated experimentally more than 20 years ago by Lüning *et al.*³, who observed mutagenic effects in the offspring of male mice which had incorporated strontium-90. These authors postulated that strontium could be present in nucleoproteins of the germ cells as a metal ion. A similar possibility was also raised recently by Riley and Willson⁴, who speculated that zinc, for instance, may be retained in the gonads and/or may be a vehicle for transporting short-lived radionuclides to critical local sites such as testicular tissue. A further hypothesis — with special reference to chemical agents — has been discussed in News and Views by Evans⁵.

We have demonstrated that yttrium-90, the daughter product of strontium-90, is

selectively retained in the gonads of male mice, thereby producing a considerable radiation dose, as measured locally by using both miniature dosimeters and determination of the yttrium-90 concentrations in the testicular tissue⁶. The β -radiation from yttrium-90 causes an initial dose rate in the testes of about 0.5 mGy h⁻¹ after injection of 37.5 kBq of yttrium-90 or strontium-90.

Radionuclides with chemical and physiological properties similar to those of yttrium are likely to behave in an analogous way. Such radionuclides include fission products in the form of rare-earth elements which, like yttrium-90, are all β -emitters, and the actinides, which belong to the same group as the rare earths. The actinides include uranium and plutonium, which are α -emitters. If such α -emitting radionuclides were to be concentrated in the testes in the same way as yttrium-90 this would result in radiation effects far greater than those of β -emitters because the α -particle energy is normally about one order of magnitude greater than that of the β -particles and produces about ten times greater internal doses for the same activity. Furthermore, the relative biological effectiveness of α -emitters per Gy absorbed dose is 10–20 times greater than that of β -emitters, resulting in an increased mutagenic effect of this order.

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Impropriety in proteins

SIR—The production of recombinant proteins from heterologous cells is limited in many cases by the failure of the protein to fold correctly into its active conformation; a common problem is that the recombinant protein forms insoluble inclusion bodies. Darby and Creighton recently suggested in *Scientific Correspondence*¹ that one reason for this behaviour is the interaction of the foreign polypeptide with some component of the cell to form a tight complex which impedes correct folding.

This suggestion is consistent with our more general proposal that several fundamental cellular processes suffer from the problem of incorrect interactions². These are defined as interactions between parts of polypeptide chains and other chemicals (either parts of the same or other polypeptides, or other chemicals such as nucleic acids or small metabolites) which lead to structures that are non-functional in the biological sense. This problem arises

because cellular processes such as protein synthesis, protein transport and the assembly and function of oligomers involve the transient exposure of interactive surfaces to the cellular environment.

The hypothesis of self-assembly³ supposes that all the interactions between surfaces exposed during such processes are both necessary and sufficient to produce the correct structure. It seems more likely that in any given interaction there will be a certain probability that incorrect structures may form. Where this probability is high the cell must reduce the formation of too large a proportion of such structures. One way that cells do this is to produce molecular chaperones, proteins that recognize and bind to transient exposed interactive surfaces in such a way that incorrect interactions are inhibited (see ref. 4).

The take-home message for biotechnologists experiencing difficulty in produc-

ing recombinant proteins in the required active form is that they should re-examine the biogenesis of the protein of interest in the cells in which it occurs naturally to determine whether molecular chaperones are involved. Where this is the case, it is worth exploring the possibility that the yield of active protein could be increased either by the co-expression of the required chaperone in the heterologous cell or by the *in vitro* addition of the chaperone to the protein as it refolds on removal of solubilizing denaturants⁵.

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Relationships from gene sequences

SIR—Analysis of gene-sequence data has led to new insights into the evolutionary relationships between organisms. These data are interpreted by inferring hypothetical point mutations and short insertions and deletions between the present-day organisms and their most recent common ancestors. The resulting phylogenetic trees represent the true evolutionary history of these organisms to the extent that one or two gene families are fully representative of the evolution of the entire genome. Clearly, a more complete description of this history requires the comparison of full genomes or at least an analysis of possible genomic evolutionary events such as transposition, duplications and other recombination-based processes.

We have recently proposed a statistical method¹, using published genetic maps and gene designations², for the comparison of gene order among bacteria. In the course of this work, we frequently encountered incompatibilities among the data sets from different bacteria. A gene from one bacteria has the same genetic designation as a completely different one from another, or genes apparently encoding homologous proteins from different bacteria have different names. Some effort has been expended to reconcile the differences between *Escherichia coli* and *Salmonella typhimurium*^{3–5}, but inconsistencies remain. Others become apparent when comparing more distant species of bacteria.

We have addressed this problem by creating a normalized gene designation database (NGDD) for the five bacteria we studied (*E. coli*, *S. typhimurium*, *B. subtilis*, *Caulobacter crescentus* and *Pseudomonas aeruginosa*).

Our work was greatly assisted by the use of the *Salmonella* gene database graciously provided by K. Sanderson⁴. In our database, containing more than 2,500 fully documented entries, gene designations are normalized to a four-letter code (three letters for the locus and one for the gene), and inconsistencies in nomenclature are cross-referenced. NGDD is available from the GenInfo Data Repository, National Center for Biotechnology Information, Building 38A, National Institutes of Health, 8600 Rockville Pike, Bethesda, Maryland 20894, USA*.

We believe that this database will be an important tool for naming newly sequenced genes from different organisms, and its availability should stimulate the reconciliation of genetic nomenclature, a problem that will only become more acute as laboratories dedicated to producing sequences on a large scale begin to produce more data.

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* Users of Internet can access the database directly at the address: NCBI.NLM.NIH.Gov. User name: Anonymous. Password: your name.

Assessing radiation dose to food

SIR—In response to concerns about the international trade of radiation-processed foods, two major international inter-comparison tests of the detection of irradiated foods will be conducted in 1990 by the Ministry of Agriculture, Fisheries and Food (Great Britain) and the International Atomic Energy Agency (Vienna). In these tests, electron spin resonance (ESR) spectrometry will be used in various laboratories for assaying irradiated food. Here, I would like to make a point concerning these measurements that was not addressed by Dodd *et al.*¹, who proposed that the radiation-absorbed dose given to meats containing bone could be estimated by the additive re-irradiation of bone samples, followed by the back-extrapolation of the ESR signal-versus-dose function. This approach not only improves the accuracy of the method but potentially eliminates the need for examination of non-irradiated samples (controls). Critical to the accuracy of the dose estimate, however, is the mathematical function used to fit the data and extrapolate to the dose axis (abscissa).

As linear² or polynomial³ functions can describe only limited portions of the ESR response to the absorbed dose in bone, such mathematical treatments of the data would not be adequate for the measurement of unknowns. In fact, when examining bone samples where the original absorbed dose and the ESR response saturation characteristics cannot be specified, large errors in the dose estimate could occur from a linear fit. The accuracy of a linear fit to the data would depend on the degree of curvature of the response for that portion of the curve. Certainly, a multiterm polynomial fit would match more closely a non-linear response, but the exponential function described here is

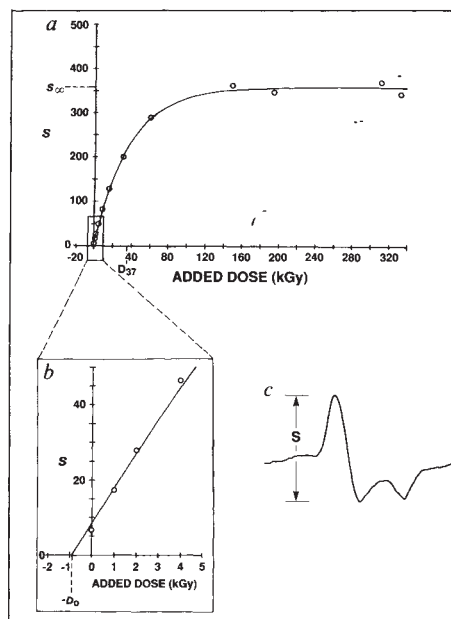
a better fit to the response of the observed data (see figure) and is a reasonable physical description of the ESR response resulting from absorption of ionizing radiation by bone^{4,5}. This approach should improve the dose estimates for bones of unknown origin and diverse radiation processing conditions by compensating for the differences in ESR signal intensity between irradiated bone samples.

The results demonstrate that the classical one-hit detector model¹ used for predicting the response of chemical sensors can also be applied to the assessment of unknown values of radiation dose given to biological tissues such as bones and teeth. In the light of the Chernobyl incident, increased emphasis has been placed on the development of dosimetry methods for radiation accident exposures in individuals or the general population. In fact, my ESR method has successfully estimated the radiation dose to workers involved in a recent accidental exposure using bone-tissue samples (manuscript in preparation). Ideally, the method could be extended to materials obtained by less invasive measures (clothing, plastic buttons and so on), which would also permit reasonable estimates of absorbed dose to radiation-sensitive organs, and thereby aid in the diagnosis and medical treatment of the victim.

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The data in *a* describe the relationship between the ESR signal intensity S (relative units) and the added gamma-ray absorbed dose D' , in kGy, for 1.00-kGy irradiated chicken bone (freeze-dried, from which a bone fragment of dimensions approximately 3×15 mm was used). The curve is a computer-generated fit using the function, $S = S_0 [1 - \exp(-(D_0 + D')/D_{37})]$; where S_0 is the ESR signal intensity at the added dose D' , S_{∞} is the signal saturation value, and D_{37} is the reciprocal of the dose at 63 per cent of the saturation value. D_0 is the original (in most cases, unknown) radiation absorbed dose. From this exponential curve-fitting of the function to the data, the calculated extrapolation value for D_0 is 0.91 ± 0.46 kGy, a reasonable estimate of the actual dose, $D_0 = 1.00$ kGy. *b*, Enlargement of the low-dose data from *a*, demonstrating the back-extrapolation method. A typical ESR spectral shape for irradiated chicken bone is shown in *c*, with the amplitude used to obtain S .

Stable taxonomy

SIR—The pressure on taxonomists to produce more stability into scientific nomenclature has generated a positive response in botanists¹ but not so far in zoologists. Botanists have established a special committee on “Names in Current Use” to produce a list, due to be published in 1991, of the approximately 36,500 currently used generic names of groups covered by the Botanical Code.

The list will be circulated widely before formal publication early in 1993 in order to obtain comments. The costs of this process have been met by the International Union of Biological Sciences, the International Association of Plant Taxonomy and the Institutes of Committee members.

The special committee will also consider proposals to change part of the International Code of Botanical Nomenclature and to protect these lists. The publication of the approximately 400,000 species names in current use will take longer, given the difficulties in obtaining money. But the committee will produce sample species lists and it hopes to cover several groups before 1993.

Obviously, zoologists would have more financial and organizational difficulties in producing a similar list, as the number of generic and species names in use in zoology (more than 200,000 and 1,500,000 respectively) is much larger. But the use of such a list is beyond doubt^{2,3}. If the botanists' example is not followed by zoologists it will not be surprising if financial support for taxonomic research is reduced.

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Impact on life

SIR—Maher and Stevenson¹ argued that giant impacts would have prevented the survival of life on the Earth's ocean floor until 3.9 Gyr ago, and on the land until 3.6 Gyr ago. Sleep *et al.*², who rejected mechanisms for annihilating life that fell short of global sterilization, considered that impacts sufficiently energetic to boil off a 200-m-deep oceanic photic zone should have continued until 3.8 Gyr ago. They argued that bottom-dwelling photosynthetic organisms would be killed as the ocean boiled away above them, while floating photosynthetic organisms fortunate enough to be mixed downwards into cooler water would be killed when returned to the ocean surface, where they would be exposed to hot, fresh rain water. It was concluded that a biosphere dependent upon photosynthetic primary

production would not have survived before 3.8 Gyr ago. We wish to point out that a different conclusion is possible, even without challenging the assumptions about impact processes.

Microorganisms exist in very large numbers, and often show great resilience to environmental stress. Consequently, the likelihood of some individuals surviving global catastrophes through special and fortuitous combinations of circumstances can be underestimated by simple models.

In the present case, we can envisage that photosynthetic prokaryotes, returned from deeper water to the new photic zone, could become associated with sea-floor sediments, or with rock surfaces, without reaching the uninhabitable uppermost part of the photic zone. It need not be assumed that such organisms would have to occur exclusively in either a free-floating or in a benthic situation. Recolonization of the photic zone and re-establishment of an ecosystem based on photoautotrophy could follow. Photosynthetic metabolism could have been evolving since before 3.8 Gyr ago.

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No barrier to exchange

SIR—Vilaro *et al.* have reported¹ the demonstration of an insulin-regulatable glucose carrier on the endothelial cells of blood capillaries which supply fat and muscle. I have no reason to question their findings, but their suggestion that such a transport system is necessary to overcome the “barrier” of the capillary wall must surely be wrong. It is generally accepted^{2,3} that water and small hydrophilic molecules of relative molecular mass 10,000 or less can diffuse freely and rapidly across the walls of normal capillaries. The micro-anatomical site of this diffusion is uncertain but some of it is believed to occur at the inter-endothelial junctions, and it has been calculated that a 40-Å separation of between 3 and 18 per cent of such junctions would account for the observed phenomena⁴.

In support of their barrier hypothesis, Vilaro *et al.* cite a paper by Karnovsky⁵. Unfortunately, this paper was concerned

mainly with the capillary barrier to the protein macromolecules horseradish peroxidase (40,000) and ferritin (500,000), and its author makes it clear that small molecules like glucose can cross the capillary wall relatively freely.

Further, it is a matter of common observation that even in the brain, where the capillaries are lined with very tightly joined endothelium, and where the glucose carrier system described by Vilaro *et al.* cannot be demonstrated, the cerebrospinal fluid nonetheless contains glucose at a concentration which is as much as 60 per cent of that in the blood.

The glucose transport system proposed by Vilaro *et al.* may well be important to fat and muscle cells, and to the function of the local vascular endothelium. But it cannot be explained directly by invoking a capillary barrier. On the basis of the available evidence, no such barrier exists.

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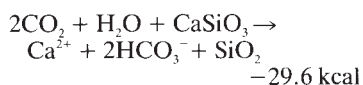
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CO₂ disposal by means of silicates

SIR—If fossil fuels are to be used on a massive scale and the greenhouse effect avoided, carbon dioxide emissions into the atmosphere must be reduced. One way to achieve this is to collect the CO₂ and to dispose of it in empty natural gas fields or the deep ocean. But such a disposal system would need a substantial amount of energy. It would be advantageous if there were an abundant mineral to which the CO₂ could be bound chemically via an exothermic reaction to form a stable, permanent substance.

It is known that about 100 million tons of carbon per year are bound by silicate-weathering throughout the world. This natural process is of a scale that would consume the CO₂ inventory of the atmosphere in 7,000 years. There are other substances to which CO₂ can bind, but the weathering reaction of calcium silicate is slightly exothermic whereas most of the others are endothermic.



One could imagine a process by which

pressurized CO₂ is pumped into a closed container containing a suspension of pulverized silicates in water. The resultant hard water and SiO₂ could be drained into the deep ocean and the process repeated, thus disposing of CO₂. This idea may not be practicable because the energy required to dig up and grind the calcium silicate may be too great and the kinetics of the above reaction may be too slow. Experimental work is necessary to determine whether this sort of scheme would be feasible in practice.

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Lithium effect

SIR—Dianna Bowles in her recent *News and Views* article¹ highlighted the role of signals in the response to trauma of the wounded plant. Two lines of research have been used to discriminate wound healing itself from the induction of a wound signal and its consequences on plant growth. First, non-traumatic treatments such as touch or wind slow the growth rate of stimulated tissues^{2,3}; at least four ‘touch-induced’ genes are involved in this process³. Second, the growth responses occurring at a distance from a wounded area can be studied, as such responses clearly do not correspond to the wound-healing process⁴.

We would like to draw attention to the fact that, in plants, lithium acts as a potent inhibitor of all these growth responses to stimuli in the range 20 μM to 1 mM. Studying the effect of injury, and of the addition of lithium, on proteinase inhibitor production would be of interest in the light of Bowles’ article. It is still not known if the molecular site of lithium action in the control of plant growth is similar to the site proposed for animal cells⁵.

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Checking out creationism

Tony Thulborn

Dictionary of Science & Creationism. By Ronald L. Ecker. *Prometheus*: 1990. Pp. 263. \$32.95, £24.50.

THE war of words between creationism and science rumbles on incessantly, plunging the general public into ever-deepening confusion about the meaning of the word 'science'. Optimists believe that the creationist crusade has suffered some resounding defeats — notably in the US state legislatures of Arkansas (1982) and Louisiana (1987) — but, in reality, these were hollow victories for science. The plain truth is that a handful of Bible-thumping extremists has corrupted what little the public knew of science in the first place. Today, nine out of ten adult Americans are "scientifically illiterate"; half of them reject outright the very thought of human evolution; and in some US states almost half of biology teachers favour the introduction of creationism in high schools.

Over the past 10 years increasing numbers of scientists have elected to stand and fight, principally by showering remedial publications upon the mountainous literature of creationism. Anyone now contemplating the overburdened bookshelves of science and pseudoscience will probably be grateful to Ronald Ecker for providing a convenient guidebook to the whole sorry mess.

Despite the title "Dictionary", Ecker's book contains only 72 text-entries. These, however, are fairly substantial essays, many of them entertaining as well as informative. In narrating the historical, legal and social aspects of creationism, Ecker — a librarian by profession — is at his best. His style is perfectly matched to the material: commendably sane and sober, but strewn with creationist gems of mind-curdling inanity. Readers may be unable to suppress their laughter on encountering the "glory meter" — an instrument for detecting whether or not you've been born again — while home gardeners will surely be diverted by creationism's official pronouncement that plants are not living organisms. Fortright souls, heading straight for the heart of the matter (under "Satan"), will learn that UFOs really do exist... on the unimpeachable authority of *Reader's Digest*. Such are the towering achievements of creation 'science'.

As a dictionary of creationism, Ecker's work is difficult to fault. Its few and minor flaws are more than offset by an excellent bibliography (more than 500 titles) and an exhaustive index. My only grumble is that Ecker concentrates almost exclusively on US creationism and its literature. His readers will never suspect that the world's

second-largest creation ministry is based in Australia, or that creationist outposts exist as far afield as Brazil, South Africa and New Zealand.

As a dictionary of science, Ecker's book is sound but unremarkable. It draws

Ecker should not be condemned for innocently reproducing the verbal blunders of science. Blame should rightly go to those scientists whose carelessness with the English language has provided creationism with so much of its ammunition. The biologist who first described a mutation as "an error" probably received a Nobel prize, instead of a well-earned reprimand. Ecker repeats the description "an error", thereby inviting more sophistry from creationism: namely, an accumulation of errors cannot possibly result in evolutionary progress.

Given the shortcomings of scientific

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Creation of the universal argument?

heavily on the second-hand generalizations of textbooks and review articles, reproducing both their strengths and, alas, their weaknesses. Thus, I regret to report, Ecker's compendium failed my preliminary test for any dictionary of science — which is to examine the entry for "homology". My standard for comparison is the *Penguin Dictionary of Biology*, which struggles with this nebulous concept for about 200 words, eventually confessing that it is "a matter of opinion". Ecker defines homology (under "anatomy") as anatomical similarity "due to common evolutionary descent". Then, under "evolution", he discloses that homologous structures are "prime evidence of shared evolutionary origin". Circularities like this are the bread-and-butter of creationism.

It is equally disappointing to read that "the evidence for biological evolution begins with the fossil record". This seemingly indestructible myth is the mainstay of creationism, which thrives on the existence of gaps in the fossil record. The primary evidence for evolution is, of course, all around us in living organisms, as Charles Darwin explained in 1859.

literature, Ecker does a creditable job. But such honest craftsmanship will not make the slightest dent in the carapace of creationism, as Martin Gardner is forced to admit in his foreword to the book. Nor, despite Gardner's hope, will it affect the thinking of politicians: they will continue to deplore the practices of Islamic fundamentalism, while encouraging the growth of Christian fundamentalism in their own back yards.

Complacent reviewers may well describe Ecker's dictionary as a valuable contribution, another handy tool for shovelling back the mire of ignorance. Seasoned campaigners, who engage creationists in classrooms and public debates, will disagree. They know, from painful experience, that a catalogue of facts and fallacies has no perceptible impact on public opinion. If science is to survive the smear-campaign of creationism, it must be made intelligible to the whole of society. And that awesome task is the responsibility of scientists — not of chivalrous bystanders like Ronald Ecker. □

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Historians and scientists

David J. Miller

Pions to Quarks: Particle Physics in the 1950s. Edited by Laurie M. Brown, Max Dresden and Lillian Hoddeson. Cambridge University Press: 1990. Pp. 734. £40, \$59.50.

"THE 1950s" are deemed to start in 1947 and end in 1963: from the discovery that pions, not muons, are the Yukawa particles that carry the nuclear interaction, to the firm establishment of SU(3) theory. This was the exploration stage of particle physics, containing a succession of surprises. Almost as soon as the pion had been identified in the cosmic radiation, the first strange-particle decays were seen. The idea of a weak interaction was developed, with associated production and strangeness. The interaction carried by the pion turned out to be too strong for the perturbation calculations that worked for electromagnetism, so dispersion relations and S-matrix theory drove out field theories for more than a decade. The biggest surprise of all was the discovery of parity violation — first hinted at by the properties of what we now call K mesons, then proved in nuclear beta-decay.

The backbone of this book is provided by the reminiscences of the physicists who did the work, complemented by contributions from professional historians of science. The right pieces of the story are told by the right people, among them Rochester, Perkins, Yang, Hofstadter, Steinberger, Pais, Pontecorvo, Treiman, Dalitz, Marshak, Schweber, Gell-Mann, Nambu and Ne'eman. Reines discusses the observation of the neutrino, Chamberlain the antiproton, and Chew hadron democracy. There is a great deal of passion in many of the accounts, and much setting the record straight — sometimes unselfishly, as when Blewett and others give credit to the self-taught Greek, Nicholas Christofilos, who invented the story focusing principle ahead of all the specialists. But there are two epic pieces of axe-grinding from great men who still do not feel that they got the credit they deserved — Piccioni, who claims he told Chamberlain and Segre how to search for the antiproton at the Berkeley Bevatron, and Sudarshan, who says that he and Marshak presented a paper on V-A weak-interaction theory at Padova a few months before the work of Feynmann and Gell-Mann appeared.

The historians' contributions are useful, particularly in putting the development of accelerators into their political context in the United States at the height of the cold war, and in their critical examination of

the written records, correcting the selective memories of the people who did the work. On the question of why the Berkeley Bevatron was built, Heilbron, a historian, lists a complex of reasons, all of which were true at the time for some of those participating in the decision. The particle physicists claim it was for the sake of pure science, in particular to find the antiproton. But the Atomic Energy Commission was also concerned to keep together Lawrence's team from the "Manhattan Engineering District" that developed the atomic bomb, and to train the next generation of weapons research physicists. At about the same time, the Lawrence Livermore Laboratory was separated from the Lawrence Berkeley Laboratory to build the first stage of a huge linear accelerator — the MTA, or Materials Testing Accelerator — intended to produce fissionable materials for weapons. Blewett, writing about how Brookhaven managed to get the Cosmotron ready before the Berkeley Bevatron, says "We knew nothing about this project; it was completely secret. . . . So far as we could see, for no visible reason, construction of the Bevatron stopped and its staff disappeared. We asked no questions, but were thankful for the decreased pressure." The historian Seidel gives more details of the MTA project, started in response to the first Soviet nuclear bomb. It never worked properly and was eventually abandoned when the Atomic

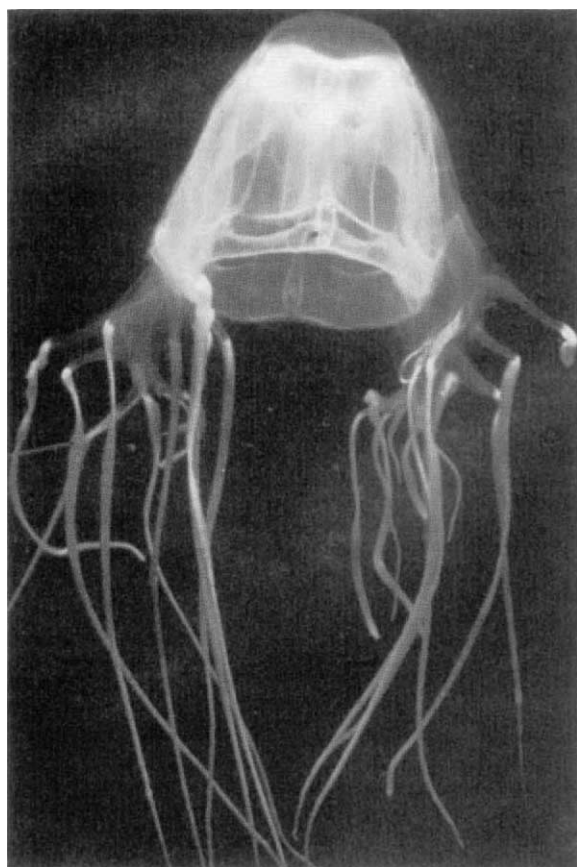
Energy Commission discovered that uranium prospecting was a more productive way of building up stocks of nuclear fuel.

Cloud chambers, emulsions and Geiger counters were the principal particle detectors for cosmic-ray work in 1947. By 1963, accelerators had almost completely taken over, the bubble chamber had arrived, spark chambers were growing in importance and new scintillating materials were being used. There are first-hand accounts of how each of these techniques evolved. Electronic computers began to appear and are mentioned in a few of the contributions, particularly in Luis Alvarez's dashing account of how he took Glazer's idea of a small 'clean' glass bubble chamber, applied large-scale engineering and organizational methods and founded the 'industrialized' particle physics of the 1960s and 70s. Galison, a historian, points out that Alvarez had to pull some of the cryogenic experts he needed out of the H-bomb programme to help him build his 72-inch liquid-hydrogen bubble chamber, starting a line of development in which particle physics laboratories still lead the world — particularly in the large-scale application of superconductivity.

This is not only an important book, it is great fun to read. Every physicist should do so. □

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Chironex fleckeri (Southcott) is probably the most dangerous marine animal known, causing death in seconds to minutes. This specimen was found near Cairns, Queensland in Australia, and is one of many species illustrated in *Dangerous and Venomous Marine Animals of the World*, whose second, revised edition has recently been published. The author, Bruce Halstead, has produced a huge and comprehensive monograph of 1,168 pages of text and 288 of illustrations. Publisher is Darwin, New Jersey. Together with P. S. Auerbach and D. Campbell, Halstead has also just written *Dangerous Marine Animals*, a lavishly illustrated colour atlas published by Wolfe Medical, price \$29.50.



Second guess

Henry Gee

Man After Man. By Dougal Dixon. Blandford: 1990. Pp.128. £14.95. To be published on 14 June.

IN THE beginning, a zoologist turned full-time dreamer called Dougal Dixon concocted *After Man*, a bestiary of 50 million years hence. It was a vision of what evolution might have achieved by then if people suddenly vanished tomorrow. Charming and insightful, *After Man* was conceptually novel and graphically witty.

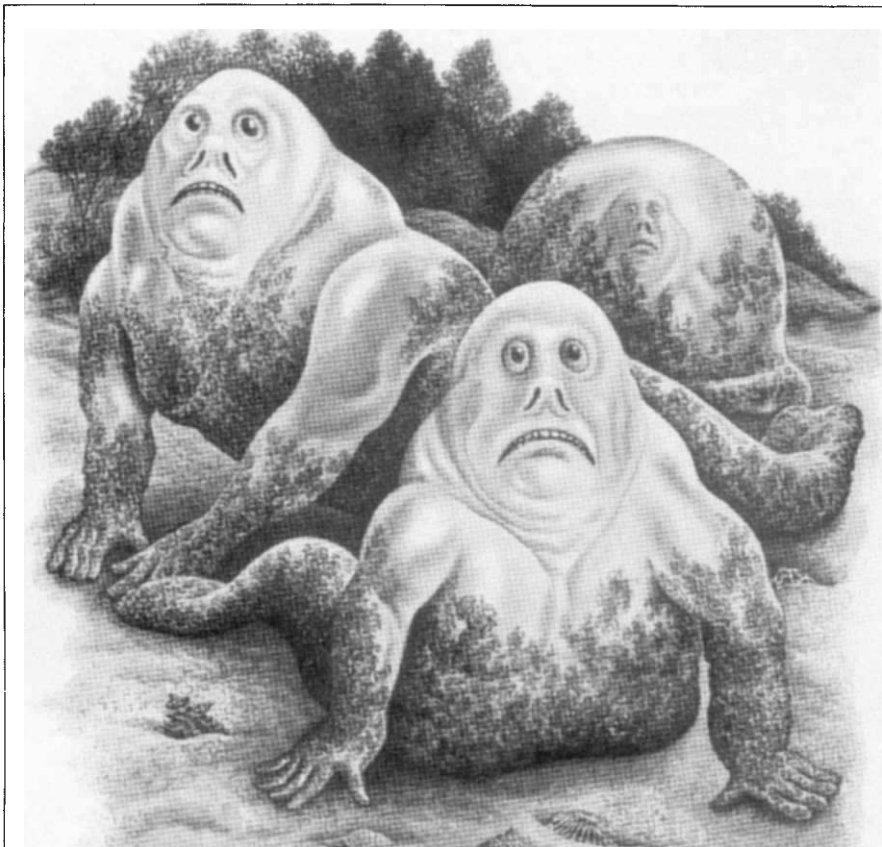
Then came *The New Dinosaurs*. This time the premise was that the famous end-Cretaceous asteroid was off-target and the dinosaurs continued to diversify. The format followed that of *After Man*, but the fit was a little procrustean. The formal names, for example, so folksy in *After Man*, began to look a little contrived. But dinosaurs and asteroids were all the rage, especially both at once, and the book still worked.

Not so *Man After Man*, Dixon's latest. The idea is an extrapolation of human evolution 5 million years into the future, and Dixon's bandwagon-of-the-year is genetic engineering. His book reads like a synopsis for a science-fiction novel by Brian Aldiss. The fact that Aldiss wrote the preface would be funny if the text were not so jejeune — lots of ideas and wizard wheezes, but the social conscience of an orang utan. The pictures, too, are lumpen and adolescent, and the *After Man*-style format completely ridiculous. The fake Latin nomenclature, like the sight of a matron squeezing into a too-small swimsuit, is pathetic and ludicrous. If only all of the pictures were gone, and Aldiss had

written a novel out of the idea, the result could have been grand.

The story goes like this. With the increase in population, human beings look to the stars for *lebensraum*. To help them, humanoids adapted for life under the sea and in the vacuum of space are engineered from spare parts. (This reminds me of the tale of the rabbi, who when confronted with the news of an impending world-wide flood, reminds his congregation that they have 48 hours to learn how to live under water.) These androids are not fertile,

And so they all plod about, each one with its face metaphorically inscribed in its chest, and a morose expression reminding us of long-lost intelligence and, no doubt, indigestion at what seems to be an exclusively cannibal feast. Then the descendants (genetically engineered) of the starfarers return, aliens in all but phylogeny, and wipe everybody out. And that just about wraps it up for humanity, except for a few fishy humanoids swimming sadly around a fashionable deep-sea vent, far from the ken of the none-too-prodigious



Ugly face of the future — life moves once again from water to land.

New Journals

This year *Nature's* annual new journals review supplement will appear in the issue of 11 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals which first appeared after June 1988, and which issued at least four separate numbers by the end of April 1990, will be considered for review. The deadline for submission is the end of June.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year.
- The main language used must be English. Translation journals in English are eligible.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title as soon as possible to: Book Review Editor, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK or 1137 National Press Building, Washington DC 20045, USA. □

but someone somewhere succeeds in establishing a viable race of underwater people.

As the cities decay into squalor and barbarism, the cream of humanity heads for the stars. The rest of the human intelligentsia, crippled by accumulated mutations, prop themselves up by prostheses until these, like the cities, fail. But good old-fashioned biotechnology saves the day, and human beings are genetically engineered (from the germ line of the underclass, who seem grateful for the opportunity to contribute) into animals, to fill the ecological niches opening in the wilderness that has overgrown most of the cities.

After five million years of going forth and multiplication, evolution would have left us with a bunch of regular anthropophagi — humanoid anteaters, sloths (ground and tree-living varieties), dolphins, mole rats, cattle, carnivores and so on.

sons, grooming themselves for a sequel.

My question is, if people could create this highly improbable mess by genetic engineering, why could they not have done it to themselves at the propped-up-by-prosthesis stage, and made life a lot more fun? Well, implies Dixon, humanity had made the world too ecologically unsound for itself by then, and special remedies were called for.

But Dixon only skirts over the ethics. Would the global environment ever deteriorate to the extent that humanity could justifiably throw morality to the wind and retrotranspose its way out of trouble? The current debates in Britain about abortion and embryo research suggest otherwise. Anyone even remotely aware of the scientific bases of these and other arguments would probably not be able to take this book very seriously. □

Henry Gee is on the editorial staff of *Nature*.

Molecular Mechanisms in Muscular Contraction

Edited by John M. Squire,
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Muscle cells provide a unique probe into the motile properties of all living systems. A full understanding of the molecular mechanisms within muscle cells is therefore important to many areas of biological research. It is now nearly 40 years since the postulation of the sliding filament model of muscle contraction yet only recently has sufficient evidence been produced to support a convincing model. This book marks a turning point in muscle research. It reviews the evidence that myosin cross-bridges do indeed swing on actin filaments during contraction, and starts to tackle the problems this raises: what are the geometrics of the attached states of cross-bridges on actin? How many states are there? What is the sequence of molecular events during muscle activation? And is the primary source of muscular force cross-bridge swinging or Helix-coil transition?

Contents: Organisation and Structure of Actomyosin Systems; J.M. Squire *et al* • The Nature of the Actin Molecule; C.E. Schutt & U. Lindberg • The Structure of F-Actin Calculated From X-Ray Fibre Diagrams and the 0.6nm Crystal Structure; K.C. Holmes *et al* • Discussion Muscle Mechanics and Biochemical Kinetics; B. Brenner • Crossbridge Patterns in Defined Static States in Insect Asynchronous Flight Muscle; M.C. Reedy *et al* • Nuclear Magnetic Resonance Studies of Calcium Modulated Proteins and Actin-Myosin Interaction; B.A. Levine *et al* • Crossbridge Movements Monitored by Extrinsic Probes; T.P. Burghardt & K. Aitai • Functional Aspects of the Myosin Rod in Contraction; W.F. Harrington *et al* • Equatorial X-Ray Diffraction Studies of Single-Skinned Muscle Fibres; L.C. Yu & R.J. Podolsky • Static and Time Resolved X-Ray Diffraction Studies of Contracting Fish Muscle; J.M. Squire & J. Harford • Index

1989 350pp 234x156 mm

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On the move

Peter Sheterline

Molecular Genetic Approaches to Protein Structure and Function. Edited by James A. Spudich. Liss: 1989. Pp.175. \$58, £40.

IF YOU are a subscriber to, or an avid reader of, the journal *Cell Motility and the Cytoskeleton*, you may pass on to the next item; this collection of review essays was published in a special issue of that journal last year. If you are still here then it may be dawning on you that although the title suggests a general review of molecular-genetic techniques in cell and developmental biology, the contents are concerned almost exclusively with examples from the study of proteins of the cytoskeleton and muscle. You will find mention of neither the elegant developmental work on *Drosophila* and *Caenorhabditis*, nor the exciting work on analysis of cell-cycle regulation in yeast.

Both these examples — there are certainly others — signal the power of molecular-genetic approaches for slicing manageable paths through the previously impenetrable undergrowth of complex cellular interactions. In these areas of cell biology recently opened up by molecular genetics, protein biochemistry has hardly had time to catch its breath. By contrast, knowledge of the cytoskeleton has been driven by studies on protein structure and function, mostly because the main proteins of the cytoskeleton constitute a significant proportion of total cell protein and are therefore relatively easy to isolate.

To those reared on strategies of grind, find, characterize and immunize, things molecular seem to be moving very fast and usually in redshift. How do molecular-genetic approaches clarify central questions pertaining to the structure and function of the cytoskeleton, questions which have matured over the past 20 years or more? Despite the panache of technique and the elegance of strategy, it is clear that the application of molecular-genetic approaches is still at an exploratory stage. The most commonly sought objectives fall into three main categories: first to express enough wild-type or engineered protein for structure and function studies; second, characterization of the phenotypic effects of expression or deletion of a protein in a suitable host cell; and third, identification and characterization of genes influencing cytoskeletal functions by conventional genetic approaches. Each of these areas is covered by one or more articles in this collection.

The expression of cytoskeletal proteins in prokaryotes, the most established vector, has turned out to be almost entirely unpredictable; gelsolin, a fragile

protein, can be isolated intact, but expression of actin, a jewel in the cytoskeletal crown, has not yet been achieved. The difficulties are not entirely unexpected and seem partly due to the lack of appropriate post-translational modification and, perhaps more darkly, to inappropriate protein-folding conditions.

Significant space is also given here to discussion of the possibilities of so-called pseudo-genetics, which involves the introduction of antisense messenger RNA into host cells to reduce their expression of selected proteins. With easy access to sequence databases and modest prices for synthetic oligonucleotides, this must be perfect entry-level 'genetics' for ex-protein biochemists.

Overall, I think that the professed objective of the editor to satisfy nascent geneticists has been successful. This success rests in part on the discussion in several papers of experimental avenues which were not directly useful or produced a quite unexpected result, but also because authors were consistent in clarifying their objectives and strategies.

Studies using cultured cells suggest that the cytoskeleton is a restless federation of structures which, by the exchange of component molecules, progressively adapts to suit prevailing conditions. The underlying mechanisms of change appear to exhibit the catastrophic behaviour of the new mathematics, and may therefore resist the linear reductionist descriptions applicable to simpler systems. There is no doubt that molecular genetics will contribute greatly to identification and structure-function relationships of component proteins, but we may have to wait for the mathematicians for any answer to the question of how the cytoskeleton works. □

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■ Also recently published, *The Cytoskeleton and Cell Motility* by T. M. Preston, C. A. King and J. S. Hyams is a short volume covering the topic for undergraduate and postgraduate students. Price is £30 hbk, £13.95 pbk, publisher is Blackie.

■ Published today is the third edition of David Aidley's textbook *The Physiology of Excitable Cells*, extensively revised and incorporating new information on the properties of single ionic channels and the molecular biology of membrane proteins. Publisher is Cambridge University Press, price £45, \$90.

■ *Turning the World Inside Out* by Robert Ehrlich contains 175 simple physics demonstrations. The book is a "treasure chest" of ideas to illustrate aspects of physical science in playful ways. Publisher is Princeton University Press, price \$50 hbk, \$14.95 pbk.

■ *Dead Heat*, by Michael Oppenheimer and Robert H. Boyle, is the latest book to describe "the race against the greenhouse effect", providing "a prescription for what America must do — before it is too late". Publisher is Basic Books, price \$19.95.

New insights into the astrophysical r-process

G. J. Mathews & J. J. Cowan

Elements heavier than iron are known to be made partly by rapid neutron capture—the astrophysical r-process. New data from nuclear experiments and astronomical observations may allow some of the proposed astrophysical sites for the r-process to be ruled out and suggest that these elements are mostly produced in type II supernovae.

It has been known for over three decades that somewhere in stars about half of the abundance of the elements heavier than iron was produced by rapid neutron-capture reactions (the r-process^{1–3}). In the r-process, neutron captures occur on time-scales much shorter than typical β -decay lifetimes. Sequential neutron captures therefore lead to the production of extremely neutron-rich isotopes. Later, these isotopes β -decay back to stable atomic nuclei as is depicted in Fig. 1.

Although the r-process contributes significantly to the elemental composition of the Galaxy, it is probably the most poorly understood of the stellar nucleosynthesis mechanisms. One reason for this is that the extremely neutron-rich isotopes formed during the r-process have been difficult to study experimentally. Another reason is that the r-process abundances are so small that they have been difficult to observe astronomically.

One important development is that β -decay rates have now been measured^{4–6} for some of the crucial unstable nuclei produced during the r-process. These measurements constrain^{7,8} the neutron density, temperature and time duration of the r-process environment. The other recent development is the measurement of stellar r-process abundances^{9–13}. Such observations show the growth of r-process abundances during the early history of the Galaxy and provide information about the kind of stellar objects that produce r-process material. These two new measurements point toward a high-neutron-density β -flow-equilibrium environment (β -flow equilibrium is the approximate equality of the product of abundance and β -decay rate for different nuclei), a neutron source that is manufactured in the star and r-process yields that track the type II supernova rate. Low-mass type II supernovae may therefore be the most likely site for the r-process.

At least ten possible sites for r-process nucleosynthesis have been proposed over the past forty years. It was initially suggested¹ that the r-process could be associated with supernovae. A number of models were developed^{2,14–18} to describe how heavy nuclei might be produced in the regions just outside neutron-rich supernova cores. Unfortunately, none of the models were able to account for the production and ejection of r-process elements

with the correct distribution to explain the Solar System abundances in a realistic and self-consistent manner.

Because of the difficulties with supernova-core models, a number of other possibilities have been studied with varying degrees of success. The proposed r-process sites can be divided into two categories: those in which the r-process elements are produced as primary products directly in a star (for example, from a photodissociated neutron-rich nucleon gas); and those in which the r-process elements are secondary (those requiring pre-existing heavy 'seed' nuclei which capture neutrons to produce r-process elements). In the former category are high-temperature, neutron-rich environments such as shock¹⁹ or jet^{20–23} ejection of material from neutron-rich supernova cores, inhomogeneous Big Bang cosmologies^{24,25}, or ejecta from binary neutron-star tidal disruption^{26–28} or coalescence²⁹. In the latter category are less neutron-rich and lower-temperature environments, such as nova outbursts³⁰, shock-induced explosive helium^{31,32} or carbon³³ burning, the core helium flash in low-mass stars³⁴, super-heated proton-rich helium burning in a pre-collapse 11- $M_{\odot}$ star³⁵ ($M_{\odot}$ = 1 solar mass), neutron-star accretion disks³⁶, or neutrino inelastic scattering in the outer envelopes of core-bounce supernovae^{37,38}. Here we summarize the available nuclear and astronomical constraints and hope to provide some insight as to which of the proposed r-process sites is most likely.

Solar System abundances and nuclear properties

Figure 2 shows the Solar System r-process abundances obtained³⁹ by subtracting the contribution from slow-neutron capture (s-process nucleosynthesis) from Solar-System abundance tables^{40–42}. The total mass fraction for r-process elements is only $\sim 10^{-7}$, that is, r-process elements account for only $\sim 10^4 M_{\odot}$ of the $\sim 10^{11} M_{\odot}$ of material in the Galaxy. The amount of r-process material produced per supernova over the history of the Galaxy (for a supernova rate⁴³ of $\sim 10^{-2} \text{ yr}^{-1}$ and an age when the Solar System formed of $\sim 10^{10} \text{ yr}$) is then only $\sim 10^4 / (10^{-2} \times 10^{10}) \approx 10^{-4} M_{\odot}$. Thus, the r-process either occurs in a small subset of supernovae, or it occurs in a small region of supernovae—smaller than the numerical zoning of many supernova models.

Another striking feature in Fig. 2 is the presence of sharp peaks in the r-process abundances near atomic mass numbers $A = 130$ and $A = 195$ (and to a lesser extent $A = 80$). Such narrow peaks suggest that most of the Solar System r-process material was produced in a single well defined environment rather than some combination of possibilities. Otherwise, averaging over environments with different temperatures and neutron densities might broaden the abundance peaks. The peaks are caused by different nuclear properties associated^{1,2} with the neutron closed shells at $N = 82$ and 126 (see Fig. 1). At high density and temperature $(n, \gamma) \leftrightarrow (\gamma, n)$ equilibrium is established. The peaks are then caused by decreased β -decay rates for nuclei near neutron closed shells (waiting points). This environment is referred to as a 'classical' r-process. Its numerical formulation is well summarized in ref. 2. For the lower-neutron-density secondary-r-process sites, the waiting-point peaks are caused⁴⁴ by diminishing neutron capture rates near neutron closed shells.

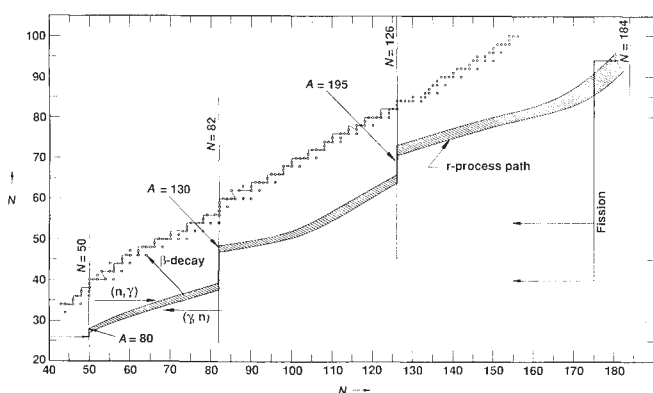


FIG. 1 A schematic view of the isotopes produced during the r-process.

The prospect for understanding the conditions for the *r*-process was recently improved by measurements of β -decay lifetimes for the waiting-point neutron-closed-shell nuclei, ^{130}Cd (ref. 4) and ^{80}Zn (refs 5, 6). It has been shown⁴ that the *r*-process abundances in the neighbourhood of these peaks are in good agreement^{7,8} with that expected from both (n, γ) and β -flow equilibrium.

This suggests that the *r*-process occurs in a classical (n, γ) equilibrium environment. We note, however, that the possibility for a secondary *r*-process may not yet be completely ruled out by these data. We have studied a secondary steady-flow *r*-process⁴⁴ in the vicinity of the $A = 80$ and 130 peaks. Using the same β -decay input data as in refs 7, 8, the abundances can be fit by adjusting the neutron capture cross-sections within their uncertainty and maintaining the neutron density above 10^{22} cm^{-3} to avoid overabundance of the doubly-closed-shell nucleus, ^{132}Sn . A definitive understanding of which environment best characterizes the *r*-process will require better neutron capture rates away from stability. In the future it may be possible to derive⁴⁵ sufficiently accurate neutron capture rates from a combination of measured level structure and β -delayed neutron emission spectra. For the present time, however, the classical (n, γ) environment seems most likely because of the remarkable agreement with observed abundances.

From the measured decay rates for waiting-point nuclei one can deduce⁴⁶ a critical neutron density at which the (n, γ) rate is equal to the β -decay rate. Using a direct-radiative-capture⁴⁶ estimate of $30 \mu\text{barn}$ for the neutron capture cross-section σ and 280 ms for the ^{130}Cd mean β -decay lifetime⁸ τ_β , gives $n_n \approx 1/(\sigma\tau_\beta) \approx 10^{20} \text{ cm}^{-3}$. For a classical *r*-process, this corresponds to the minimum neutron density necessary to maintain the waiting-point condition⁴⁴. For a secondary *r*-process this represents an estimate of the neutron density required for neutron capture to reach the waiting point before β -decay. It is difficult⁴⁷ for most secondary *r*-process models to obtain neutron densities in excess of this critical value, supporting the notion

of a classical primary *r*-process.

The critical neutron density also determines the conditions at which the *r*-process will no longer occur, or the freezeout condition. Figure 3 shows allowed values of neutron density as a function of temperature^{7,8} and the conditions necessary⁴⁴ for the waiting-point approximation to be valid. Neutron source reactions are quite temperature sensitive. Therefore, the *r*-process probably begins at a higher temperature and significantly higher neutron density than the critical neutron density as depicted in Fig. 3. As the environment cools and the neutron density decreases the final observed abundances result from the conditions just before the neutron density drops below the critical value. This explains why the *r*-process peaks are so sharp. Although the neutron density may have been higher during the *r*-process, the final observed abundances only reflect the conditions during the short time just before the neutron density dropped below the critical value.

The fact that the *r*-process seems to last long enough for β -decay flow to equilibrate allows for an estimate of the minimum time duration for the *r*-process. β -flow equilibrium requires that the *r*-process continue for at least about twice the mean β -decay lifetime of the slowest waiting-point nucleus (^{80}Zn , $\tau_\beta = 540 \text{ ms}$, refs 5, 6). Thus, the *r*-process probably lasts for at least $\sim 1 \text{ s}$. This is comparable to the time for the *r*-process to flow from iron to actinide nuclei ($\sim 2 \text{ s}$, refs 7, 8), and to the time required ($\sim 1 \text{ s}$) to shift the *s*-process peaks to *r*-process peaks in a secondary *r*-process³².

The timescale could, however, be shorter if the neutron density is a rapidly decreasing function of temperature and time, that is, the *r*-process path may begin further from stability where shorter β -decay lifetimes imply a shorter timescale for equilibration.

The new nuclear data have provided valuable clues to the nature of the *r*-process site. Although much more data for nuclei along the *r*-process path are needed, it seems most likely that the *r*-process occurs in a classical primary environment. We also

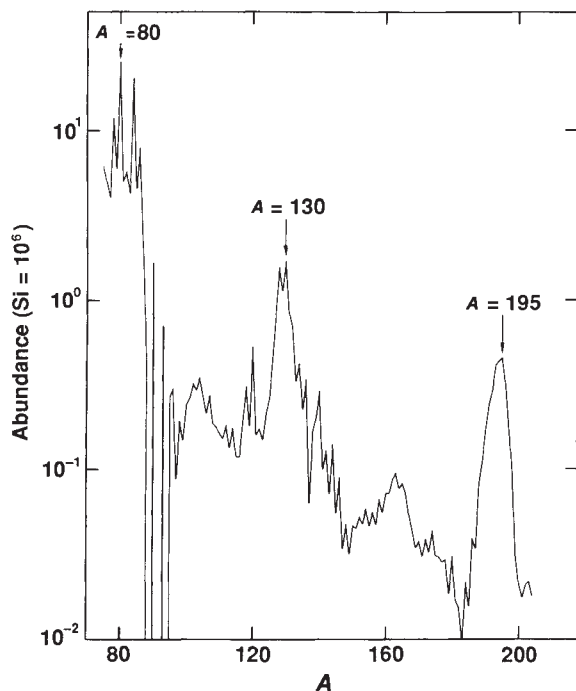


FIG. 2 An example of Solar System *r*-process abundances from the analysis of ref. 39.

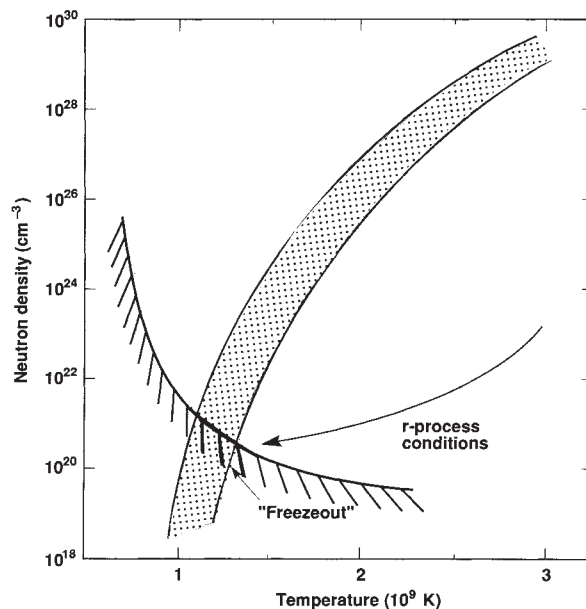


FIG. 3 The dotted area contains the neutron densities and temperatures consistent with observed *r*-process peaks and measured β -decay rates from refs 7 and 8. The hatched region shows the conditions for which the classical waiting-point approximation is valid⁴⁴. The uncertainty in the allowed conditions is the result of the uncertainty in the nuclear-mass predictions away from stability. The thick arrow is a schematic illustration of the temperatures and densities produced in an *r*-process environment before freezeout at $n_n \approx 10^{20}$ and $T \approx 10^9 \text{ K}$.

have a clearer picture of the neutron density ($\sim 10^{20} \text{ cm}^{-3}$), temperature ($\sim 10^9 \text{ K}$) and timescale ($\sim 1 \text{ s}$) for the termination of the r-process.

Astronomical data and galactic evolution

Recently, numerous measurements have been made⁹⁻¹² of heavy-element abundances on the surface of metal-poor stars. Such data display the enrichment of r-process abundances during the history of the Galaxy. Abundance correlations of nearly pure r-process elements (like europium) can now be constructed with respect to iron 'seed' material over several orders of magnitude in abundance.

One of the first exciting discoveries¹³ was that the r-process abundances relative to iron in metal-poor stars look much like the Solar System r-process distribution. This important result confirms the notion that the r-process is a unique event which always produces an abundance distribution similar to that observed in the Solar System.

The correlation of heavy-element abundances with iron are also useful for two other reasons. The astrophysical sites for the production of iron are reasonably well understood^{48,49} to be primary. Also, the abundance of iron over the history of the Galaxy has been constructed^{50,51} from studies of iron abundance as a function of stellar age. Therefore, correlations of r-process elements with respect to iron also give information of the time history of the r-process.

We know the nucleosynthesis mechanism for iron from the fact that the light curve from all types of supernovae (Ia, Ib and type II) are powered by the decay of radioactive ^{56}Ni to ^{56}Fe . This fact was established⁵² by the observation of the growth of the Co absorption line from ^{56}Ni decay in the spectra of supernovae. There are theoretical reasons to expect that type Ia supernovae (and perhaps Ib supernovae as well) are produced by the ignition of a thermonuclear runaway that produces $\sim 0.5 M_{\odot}$ of ^{56}Fe per event. The production of ^{56}Fe in type II supernovae was recently calibrated by the explosion of SN1987A in the Large Magellanic Cloud. The light curve indicates⁵³ a yield of $0.07 M_{\odot}$ of Fe which is in good agreement with theoretical predictions⁵². Thus, iron is a primary element. This makes correlations of iron with r-process elements useful. Primary r-process elements will grow at a similar rate to iron during the history of the Galaxy. Secondary r-process elements will be delayed until iron has grown to a significant fraction of its present abundance.

We have constructed models⁵⁴ for the evolution of the iron and r-process abundances based on various rates⁵⁵ of star formation and the relative probability of forming stars of different masses and estimates⁵⁶ of stellar lifetimes. Our models for iron production from supernovae are constrained by observed supernova rates⁴³ and the iron abundance as a function of stellar age^{50,51}.

Figure 4 shows the observed correlation⁹⁻¹¹ of the logarithm of the Eu/Fe ratio relative to its Solar System value (designated by $[\text{Eu/Fe}]$) as a function of $[\text{Fe/H}]$ ($\log(\text{Fe/H}) - \log(\text{Fe/H})_{\odot}$) compared with a number of r-process scenarios. Europium is a good indicator of the r-process because it is about 90% of pure r-process origin in the Solar System³⁹.

The fact that the observed $[\text{Eu/Fe}]$ ratio is nearly constant for much of the range of $[\text{Fe/H}]$ indicates that the r-process elements have increased along with iron over the history of the Galaxy. This rules out a pregalactic primordial source for the bulk of the r-process which appears as the dashed line labelled 'Primordial' in Fig. 4. Nevertheless, the possibility of some primordial r-process ($< 10^{-3}$ of the solar abundance) remains an intriguing possibility^{24,25} as a signature of baryon-number inhomogeneities during the Big Bang.

The dashed line on Fig. 4 labelled 'All SN II' shows a calculation in which both the r-process elements and Fe are initially produced as primary elements from type II supernovae. The decrease in the $[\text{Eu/Fe}]$ ratio as $[\text{Fe/H}]$ increases from

-2.0 to $+1.0$ is a consequence of the fact that iron is also produced by type I supernovae, which eject their iron later.

There may be a decrease¹¹ in $[\text{Eu/Fe}]$ for $[\text{Fe/H}] < -2.0$ (but see ref. 12). This drop may indicate that the r-process begins a little bit later than the processes that form iron. As iron is first produced in massive stars, the fall off in $[\text{Eu/Fe}]$ suggests that r-process elements are produced in lower-mass stars which explode later. The solid line in Fig. 4 labelled 'Low-mass SN II' is from a model in which the r-process occurs in type II supernovae with initial masses of $10-11 M_{\odot}$.

The fact that low-mass stars may fit the abundances best is consistent with results from computer models^{52,57} for core-bounce supernovae. Low-mass supernovae explode and eject neutron-rich core material more easily. This conclusion is supported by SN1987A, which had a high-mass progenitor, and ejected only a small amount⁵³ of ^{56}Ni . Thus, very little neutron-rich core material (which lies below the ^{56}Ni) could have been ejected either.

Nevertheless, this argument is model dependent. For example, if the production of iron in type II supernovae is skewed toward more massive stars, this would decrease the Eu/Fe ratio early on without constraining the r-process to low-mass supernovae. Also we note that the precise mass range for the r-process supernovae is dependent on stellar age estimates. In some more recent models low-mass stars evolve more quickly relative to high-mass stars than our estimates. This could imply a lower mass range for r-process supernovae. In subsequent, more detailed calculations we have found that these two effects approximately cancel.

The dot-dashed line on Fig. 4 labelled 'neutron star binaries' shows a calculation of the production of $[\text{Eu/Fe}]$ from the merger²⁶⁻²⁸ of two neutron stars. The r-process is delayed by the time required for gravitational radiation to bring the system to merger. We estimate this time from the minimum orbital separation that allows two massive stars to evolve to a neutron star. When either star is in the red-giant phase the other star must be far enough away that the helium envelope of the red giant is not lost by overflow onto the more compact companion star. Otherwise, the stars would evolve to carbon-oxygen white dwarfs rather than to neutron stars⁵⁸.

From this we deduce that the minimum orbital separation of a neutron-star binary when formed is ~ 2.5 solar radii. This is comparable to the separation of the binary-pulsar candidate, PSR - 1913 + 16, which is at a separation distance of ~ 1.5 solar radii. The time for gravitational radiation damping of this orbit

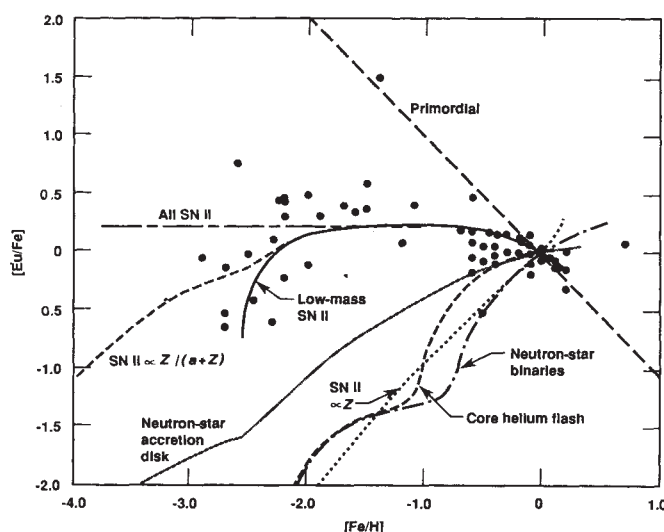


FIG. 4 Correlation of $[\text{Eu/Fe}]$ with $[\text{Fe/H}]$. The points are data from refs 9-11. See text for details.

is $\sim 10^9$ yr which is too long to reproduce the observations. But if the binary is formed in a high-eccentricity orbit, the orbital decay lifetime could be an order of magnitude less. The [Eu/Fe] curve might then better represent the data. Also, the timescale for damping of a system comprising a black hole and a neutron star would be more rapid than for a neutron-star binary^{26,27}.

The fact that [Eu/Fe] remains very close to but slightly in excess of the solar ratio as the iron abundance decreases by two orders of magnitude is evidence against a secondary r-process in which the yield is proportional to the pre-existing heavy-element abundance. The dotted line labelled 'SN II $\propto Z$ ' in Fig. 4 shows a model for such an r-process in type II supernovae. Independent of model parameters, the Eu/Fe ratio is always below the Solar System ratio. But even a secondary r-process may produce yields that are independent of the amount of pre-existing seed material as long as there is enough seed material available. The r-process abundance yields depend on the product of the neutron density and the abundance of heavy seed material. The neutron abundance, however, depends on the competition between neutron production and absorption by capture reactions. Because the heavy nuclei have much larger neutron capture cross-sections, the average absorption cross-section is dominated by heavy elements once the abundance of iron and other heavy 'seed' nuclei has increased above a certain threshold. Increased r-process yields from increased seed material can therefore be cancelled by the decreased neutron density from neutron absorption and so final r-process yields only depend on the neutron-source abundance.

For a secondary r-process to be metallicity-independent it is therefore necessary for the neutron source to be independent of metallicity. Examples of such sources are the $^{13}\text{C}(\alpha, n)^{16}\text{O}$ reaction which uses ^{13}C that has been manufactured in the star by mixing hydrogen with ^{12}C produced in the helium-burning zone⁴⁷, or neutrino-induced neutron emission from carbon or helium in the outer envelopes of core-bounce supernovae^{37,38}.

One can estimate from the neutron capture cross-sections that the initial seed abundance must be [Fe/H] ≈ -3 before the r-process yields are metallicity-independent. The dashed line in Fig. 4 labelled 'SN II $\propto Z/(a+Z)$ ' shows a good fit to the observed [Eu/Fe] ratio based on a secondary r-process in which

neutron capture on primary and secondary nuclei has been included.

A core-helium-flash model³⁴ for the r-process in low-mass stars ($\sim 1-3 M_{\odot}$) is shown by a dashed line in Fig. 4. The time delay for such stars to contribute is too slow compared to the growth of iron. Figure 4 also shows a model³⁶ in which r-process material is ejected from neutron-star accretion disks. If the accretion occurs over a timescale comparable to the galactic age, then the rate of ejection of r-process material becomes proportional to the total number of neutron-star remnants. This scenario does not well fit the Eu/Fe ratio although it may be possible to shorten the accretion time and improve the agreement.

Discussion

There are still large uncertainties in the available constraints on the astrophysical site for r-process nucleosynthesis. On the basis of recent β -decay measurements, the r-process probably occurs in a high neutron density ($n_n \geq 10^{20} \text{ cm}^{-3}$) classical (n, γ) equilibrium environment. The observed stellar abundances indicate that the r-process probably occurs in a single unique environment. Because the [Eu/Fe] ratio slightly exceeds the solar ratio for $-2.0 < [\text{Fe}/\text{H}] < 0.0$, the r-process is probably associated with type II supernovae. The apparent decrease in [Eu/Fe] for $[\text{Fe}/\text{H}] \leq -2.5$ suggests that the r-process may be a primary event in low-mass type II supernovae or a secondary event with a neutron source that does not require pre-existing seed material.

Of the list of possible sites of r-process nucleosynthesis those that can generate the highest neutron densities (such as ejected core material from low-mass supernovae) are the most favoured whereas those with lower neutron densities or large progenitor evolution times (like the helium core flash in low-mass stars) are least favoured. For all of these reasons we conclude that the ejection of neutron-rich core material from low-mass type-II supernovae may be the most likely site, but emphasize the need for more nuclear and astronomical data. $\square$

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ACKNOWLEDGEMENTS. We acknowledge useful discussions with G. Bazan, J. Lattimer, B. S. Meyer & F.-K. Thielemann. This work was performed under the auspices of the US Department of Energy at Lawrence Livermore National Laboratory and was supported by the NSF at the University of Oklahoma.

Identification by anti-idiotypic antibodies of an intracellular membrane protein that recognizes a mammalian endoplasmic reticulum retention signal

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Monoclonal antibodies were raised against antibodies to distinct carboxy-terminal KDEL sequences of two soluble, resident endoplasmic reticulum proteins. These anti-idiotypic reagents recognize an intrinsic membrane protein with characteristics expected of a receptor responsible for the recognition and return of resident proteins to the endoplasmic reticulum.

EUKARYOTIC secretory proteins begin their journey out of the cell by co-translational translocation into the lumen of the endoplasmic reticulum (ER)¹. Vesicular transport² of these soluble proteins to the Golgi apparatus seems to be by a non-specific fluid phase transport that does not require a specific signal³. The presence of several resident soluble proteins in the lumen of the ER provides an apparent exception to this hypothesis of nonspecific fluid phase transport⁴. Several of these resident proteins mediate the proper assembly and folding of newly synthesized proteins. They include the immunoglobulin heavy chain-binding protein (BiP), which apparently functions in protein oligomerization⁵, and protein disulphide isomerase (PDI), which catalyses the rearrangement of disulphide bonds⁶. These retained soluble ER proteins are not a minor population; from data in the literature^{7,8} we calculate that PDI is a very abundant protein of the ER, present at a concentration of >400 μ M.

A clue to the mechanism of retention in the ER came from the finding that BiP and PDI share a carboxy-terminal sequence (Table 1), Lys-Asp-Glu-Leu (KDEL) with other resident ER proteins including glucose-regulated protein 94 (GRP 94) (ref. 9). In addition, a secreted soluble protein, lysozyme, can be converted into a retained protein of the ER by adding the last six amino acids of BiP, Ser-Glu-Lys-Asp-Glu-Leu, to the carboxy terminus. Addition of a further two amino acids (Gly-Leu; GL) to the carboxy terminus results in secretion of the mutant lysozyme, as do numerous other changes in the KDEL terminus¹⁰. Thus, the presence of KDEL at the carboxy terminus is sufficient for the retention of a protein in the ER. A similar retention system functions, albeit less efficiently, in the yeast *Saccharomyces cerevisiae*¹¹, although a different recognition signal, His-Asp-Glu-Leu (HDEL), is used.

The retention of resident proteins bearing the KDEL signal by interaction with a static receptor within the ER seems unlikely: the proteins that must be retained by this receptor are far more abundant than any single candidate receptor polypeptide. It has been suggested that the receptor might function in a distal compartment by recognizing the KDEL signal on escaping resident proteins and mediating their return to the ER¹⁰. Microinjection experiments¹² demonstrate that the presence of

a KDEL tail on BiP does not significantly alter its rate of diffusion in the ER from that of BiP bearing the unrecognized KDELGL tail. Both diffuse more rapidly than the integral membrane protein, haemagglutinin, but more slowly than the secreted protein, albumin. Hence, the KDEL signal is not continually and tightly bound to a component of the ER membrane, although proteins bearing this signal probably interact weakly within the ER. Proteins with the KDEL signal do leave the ER transiently, as shown by the modification of a cathepsin D hybrid protein bearing the KDEL tail¹³. Thus, resident ER proteins presumably pass through a distal compartment that is proximal to the classical Golgi apparatus before returning to the ER⁴. Compartments have been identified between the ER and classical Golgi by a variety of approaches¹⁴⁻¹⁶. Although the relationship between these structures remains unclear, we will refer to them collectively as the salvage compartment⁴.

The identification of a receptor for the KDEL signal must rest on several assumptions about its properties. First, we assumed that all KDEL signals must be recognized by a single receptor species that does not bind unrecognized sequences such as K...GL. Second, we assumed that the receptor is a recycling protein and must have two distinct binding states. While functioning, the receptor must alternate between a high-affinity state that recognizes escaped KDEL-bearing proteins and a low-affinity state that releases them into the lumen of the ER. Conventional ligand affinity methods for receptor identification are problematic because the ionic environment of the ER and salvage compartment are not well defined. We made broad assumptions about receptor localization. The receptor could be present in the ER, in transitional elements, in the salvage compartment, in proximal parts of the Golgi apparatus and in transport vesicles between these compartments. For these reasons we used an immunological approach to search for a protein with the properties required of the receptor.

Monoclonal internal image anti-idiotypic antibodies are powerful tools for the identification and characterization of the participants in a receptor-ligand interaction^{17,18}. Peptides containing the KDEL signal (Table 1) can be used to raise anti-KDEL antibodies that recognize this signal at the carboxy terminus of ligands. Internal image anti-idiotypic antibodies¹⁸⁻²⁰ raised against these anti-KDEL antibodies should then recognize the receptor by its KDEL-binding site. We raised such anti-idiotypic antibodies and demonstrate here that they recognize an intrinsic membrane protein that is a candidate for the KDEL receptor.

Antibody generation

Synthetic peptides corresponding to a variety of carboxy-terminal sequences (Table 1) were used to generate polyclonal and monoclonal antibodies. Rabbit antisera to some of these peptides have already been shown to recognize the corresponding ER proteins and to label the ER by electron microscope (EM) immunocytochemistry²¹; a more complete characterization of these antibodies will be presented elsewhere (S.F., J.T.

‡ To whom correspondence should be addressed.

TABLE 1 Sequences of synthetic peptides used

Name	Sequence	Origin
KAVK	KAVKDEL	short PDI
KDDD	KDDQKAVKDEL	long PDI
KETE	KETEKESTEKDEL	GRP 94
KEED	KEEDTSEKDEL	long BiP
KSEK	KSEKDEL	short BiP
KEEE	KEEESPGQAKDEL	Calreticulin
K...GL	KEEDTSEKDELGL	nonrecognized tail
KX ₅	KXXXXXKDEL	mixed tail
HDEL	KDDGDYFEHDEL	<i>S. cerevisiae</i> BiP

Peptides were synthesized in an automated continuous flow instrument using Fmoc chemistry²⁹ and purified by reverse phase HPLC. The peptide KXXXXXKDEL consists of a mixture of analogues containing any one of alanine, aspartic acid, histidine, glutamine, leucine, tyrosine or lysine at each of the positions X. KDEL-terminated peptides are identified throughout by their unique amino-terminal residues. Single-letter amino-acid code used.

and D.V., manuscript in preparation). An important assumption of the anti-idiotypic approach is that the first round antibodies recognize the KDEL retention signal itself on resident ER proteins. As the signal specificity lies within the last four residues, recognition by our first round antibodies must be dependent on the carboxy terminus. A monoclonal anti-PDI tail antibody was generated by immunization with peptide KDDD (Table 1). This IgG antibody (1D3) recognizes a double band of relative molecular mass ~55,000 (M_r ~55K) in western blots, consisting of PDI and calreticulin²², labels the ER by EM

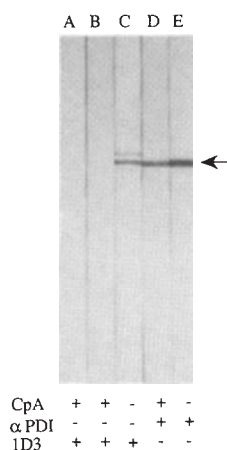


FIG. 1 Monoclonal antibody, 1D3, recognizes a carboxy-terminal determinant on an ER protein, PDI. Western blot using monoclonal antibody 1D3 (lanes A, B, C) or rabbit anti-PDI antiserum (lanes D, E) on total cellular protein before (lanes C, E) and after treatment with carboxypeptidase A at low concentration (lane A) or at high concentration (lanes B, D). The arrow identifies PDI.

METHODS. VG mouse hybridoma total cell protein was separated by gel electrophoresis³⁰ and electrophoretically transferred to nitrocellulose using a Genie electroblotter (Idea Scientific Inc.) according to the manufacturers instructions. Strips from a single blot were washed in 2% (v/v) Tween-20 in 25 mM Tris-HCl buffer 150 mM NaCl (TN), pH 7.6, and incubated overnight at 37 °C in 25 ml of 2% Tween-20 TN pH 5.5 buffer, other incubations contained a 1:250 or a 1:2500 dilution of carboxypeptidase A (Sigma). The filters were then washed in 2% Tween-20 TN pH 7.6, blocked with BLOTTO³¹, and probed using either monoclonal antibody 1D3 (prepared against KDDD peptide coupled to Keyhole limpet hemocyanin (KLH)) or a rabbit antiserum to PDI (rabbit immunized as described²¹ with protein purified by the method of Lambert and Freedman³²) followed by appropriate affinity-purified alkaline phosphatase-conjugated goat second antibodies³³. Two-dimensional gel electrophoresis followed by western blotting with anti-KEEE antiserum confirmed that the second band recognized by 1D3 is calreticulin (lane C).

immunocytochemistry²¹ and gives a clear reticular pattern by immunofluorescence (Fig. 7h). Figure 1 confirms that the signal recognized by 1D3 can be removed from PDI by gentle incubation with carboxypeptidase A under conditions that do not change the apparent protein pattern of the blotted lysate and do not affect the recognition of PDI by a polyclonal antiserum raised against rat PDI. Thus, 1D3 recognition depends on carboxy-terminal residues. Taken in conjunction with solid phase assays on a range of synthetic peptides (data not shown), this result leads us to conclude that 1D3 recognizes the retention signal in PDI and calreticulin. Similarly, polyclonal antisera to the KETE peptide (Table 1) recognize specifically the carboxy-terminal residues of BiP, PDI, GRP 94 and other proteins of the ER (S.F., J.T. and D.V., manuscript in preparation).

Two approaches were used to prepare anti-idiotypic antibodies to the anti-tail antibodies. First, monoclonal anti-idiotypic antibodies were generated by the method of paired *in vitro* immunization¹⁸ using unconjugated KETE peptide as the starting immunogen. The second-round hybridomas were screened for ER- or Golgi-related staining patterns by indirect immunofluorescence. Two wells were selected for further characterization, both of which yielded stable IgM-secreting hybridoma cell lines after cloning in agarose. The results obtained with the line designated 5D3 are described below. Second, syngeneic mice were immunized with fixed cells of the 1D3 hybridoma cell line. Hybridomas resulting from this *in vivo* immunization were screened for reactivity with anti-KDEL antibodies (rabbit anti-KAVK). Of 256 hybrid-containing wells, 15 gave a strong reaction. The results for a stable, cloned IgG-secreting line from one of these, 9D6, are described below.

Anti-anti-KDEL antibodies

Immunoblotting of lysates of control murine hybridoma cells (VG cells) revealed a major immunoreactive band of 72K with 5D3 and 9D6 (Fig. 2a). This 72K antigen remained with the membrane pellet after carbonate-washing of microsomes up to pH 11 (Fig. 2e). The 72K antigen also partitioned into the detergent phase on separation of a TX-114 lysate of VG cells (not shown). Thus, the antigen is presumably an integral membrane protein²³. Protease protection experiments revealed that a 54K immunoreactive fragment remained resistant to protease treatment of intact VG cell microsomes (Fig. 2c). Neither band was present when protease treatment followed detergent lysis, and blotting with anti-PDI showed that this luminal ER protein was completely protected under the conditions of Fig. 2c, lane 2 (not shown). Immunoblotting with 5D3 showed that the antigen was present in all the vertebrate species tested but not in *S. cerevisiae* (data not shown).

Radio-labelled antigen could be immunoprecipitated from [³⁵S]methionine-labelled lysates of 5D3 or 9D6 hybridoma cells (Fig. 2b) or from lysates of isolated microsomes (data not shown) simply by the addition of the appropriate second antibody and fixed *Staphylococcus aureus* cells. Pulse-chase analysis (data not shown) demonstrated that this complex existed intracellularly before cell lysis. The antigen immunoprecipitated with 5D3 was recognized in immunoblots by 9D6 and the antigen immunoprecipitated with 9D6 was recognized by 5D3 (Fig. 2b).

A third anti-idiotypic response was produced when 1D3 ascites was generated in unirradiated syngeneic mice. The ascites contained an IgM that was reactive with the same 72K band by western blotting (Fig. 2d, lane 5), but which was absent from 1D3 culture supernatant (Fig. 2d, lane 4). Presumably this is because the intact immune system of the tumour-bearing mice responds to the presence of high levels of 1D3 immunoglobulin by producing a primary (IgM) anti-idiotypic response, which includes the internal image anti-idiotypic specificity capable of recognizing the putative receptor. Taken together, these results indicate that three different routes of anti-idiotypic production generate antibodies that recognize the same 72K conserved membrane protein.

The anti-idiotypic antibodies recognize a common idiotope in antisera against KDEL peptides (Fig. 3). For example, 5D3 not only recognizes a rabbit antibody raised against the original KETE peptide but also rabbit antibodies raised against other KDEL peptides (Fig. 3a). The complementary assay (Fig. 3b) showed that the rabbit anti-KDEL antisera recognize 9D6 and 5D3 immunoglobulins more strongly than either the monoclonal antibody, 1D3, or another control antibody. No reactivity was seen against rabbit antibodies raised against two irrelevant peptides (Fig. 3a) or preimmune rabbit serum (Fig. 3a and b). This cross-specificity is particularly striking as some of the individual anti-tail antibodies show a much narrower range of reactivity. For example, a rabbit anti-KDDD antiserum that recognizes PDI does not recognize BiP, although rabbit anti-KEED, which recognizes BiP, does not recognize PDI (D.V., J.T. and S.F. manuscript in preparation).

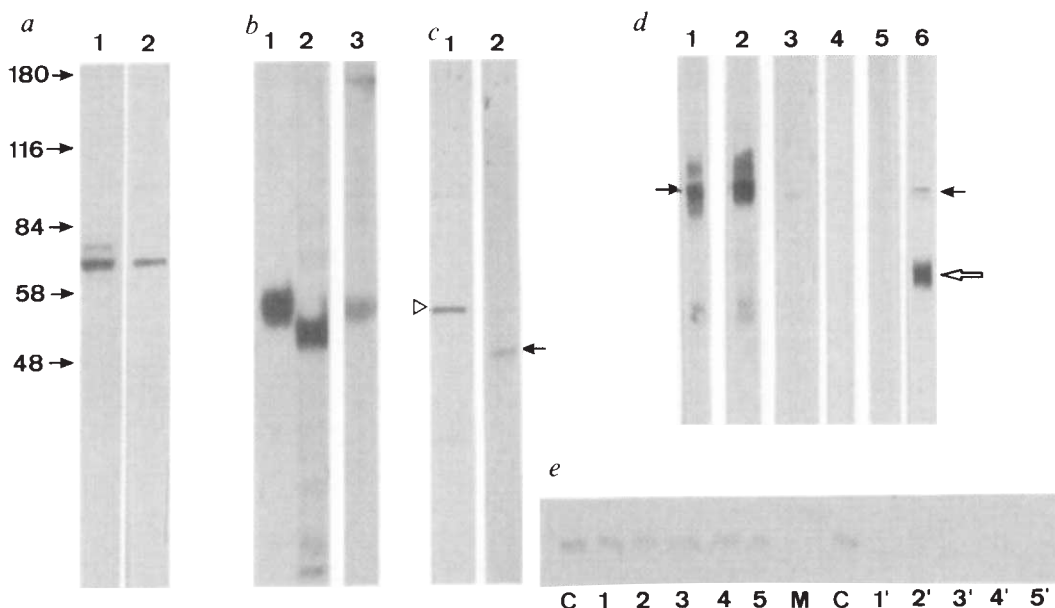
If the anti-idiotypes mimic the common structure of KDEL tails recognized by the receptor, then antibodies raised against 5D3 or 9D6 immunoglobulins should show the broad KDEL tail specificity of the receptor itself. This would also be proof that 5D3 and 9D6 are internal image anti-idiotypic antibodies. Evidence for this was obtained from a third round of immunization. Anti-5D3 immunoglobulin sera were generated in

BALB/c mice by immunization with fixed 5D3 hybridoma cells. This antisera specifically recognized peptides terminated with KDEL and not those terminated with K...GL (Fig. 4a). Furthermore, this recognition extended to KDEL peptides other than the original antigen. A comparable primary IgM response was found in the serum or ascites from nonirradiated mice bearing either anti-idiotypic hybridoma as an ascitic tumour (Fig. 4b). These results demonstrate that anti-idiotypes elicited by 5D3 or 9D6 recognize the structural feature common to the KDEL tails. Hence, the protein identified by 5D3 and 9D6 should also recognize the KDEL signal on a variety of proteins, which makes it a good candidate for the role of KDEL receptor.

Anti-idiotypic secreting hybridoma cells

The properties of the 5D3 and 9D6 cells themselves lend further support to the assignment of the 72K antigen as the KDEL receptor. We have already demonstrated that the antigen interacts with the antibodies intracellularly, so it is not surprising that the hybridomas grew poorly, cloned inefficiently and secreted only small amounts of correctly assembled immunoglobulins. Nevertheless, the cells survived and therefore must have compensated for the presence of anti-receptor antibody. There are two mechanisms by which antibody interference with

FIG. 2 Both monoclonal anti-idiotypic antibodies recognize the same integral membrane protein. The total post-nuclear lysate from the mouse hybridoma cell line VG was blotted with the monoclonal antibodies 5D3 (a, lane 1) and 9D6 (a, lane 2). M_r (in K) marker positions for a are indicated to the left. Antigen was immuno-precipitated from [35 S]methionine-labelled 9D6 (b, lane 1) or 5D3 (b, lane 2) cell lysates. The cross reactivity of the 5D3 and 9D6 antigens is demonstrated by the isolation of the immunoglobulin-antigen complex from supernatant of 9D6 hybridomas with fixed *S. aureus* cells and immunoblotting with 5D3 (b, lane 3). VG microsomes treated with proteinase K and blotted with 5D3 show that a cytoplasmic portion of the antigen is accessible to cleavage and that the antigenic site is contained within the protected luminal domain (c, lane 2, solid arrow; undigested antigen, c, lane 1, open arrow). Blotting of an equal amount of protein from total post-nuclear lysates from 5D3 (d, lane 1), 9D6 (d, lane 2) or VG (d, lane 3) with the monoclonal antibody 5D3 shows the increased level of antigen (solid arrows) expression in the anti-idiotypic hybridomas. VG lysate was immunoblotted with 1D3 ascites using a μ -chain-specific antibody to show IgM reactivity (d, lane 5), which was absent when 1D3 supernatant was used (d, lane 4). VG lysate was also western blotted with polyclonal mouse antisera raised against the isolated 5D3 antigen (d, lane 6). The position of the mouse heavy chain in VG cell lysates is marked by the open arrow. e, Demonstration of the membrane-associated nature of the 5D3/9D6 antigen by carbonate washing of VG microsomes at various pH (lane 1, pH 11.6; 2, pH 11.0; 3, pH 10.5; 4, pH 10.0; 5, pH 9.5) followed by western blotting of the pellet (lanes 1–5) or supernatant (lanes 1'–5') with 5D3. The 84K marker (lane M) and noncarbonate-treated microsomes (lane C) are also shown.



METHODS. Monoclonal antibody 5D3 was produced by paired *in vitro* immunization¹⁸ using 100 μ g unconjugated HPLC-purified KETE peptide as starting antigen. Monoclonal antibody 9D6 was produced by fusing Ag8 myeloma cells to spleen cells from a BALB/c mouse immunized with washed 3% (w/v) paraformaldehyde-fixed 1D3 (anti-PDI tail) hybridoma cells³⁴. Washed VG cells at 10^7 ml⁻¹ were lysed in lysis buffer (LB; calcium/mag-

nesium-free PBS containing 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1% aprotinin (Sigma), 1 mM EDTA and 10 mM sodium azide) and the post-nuclear fraction separated on 10% polyacrylamide gels. Transfer and western blotting was carried out as in Fig. 1 using appropriate affinity-purified isotype-specific goat anti-mouse alkaline phosphatase conjugates (Zymed Inc., San Francisco). Microsomes were prepared from VG cells by resuspending a washed cell pellet in 0.25 M sucrose containing 3 mM imidazole, PMSF and aprotinin, 1 mM MgCl₂, 10 mM CaCl₂ and passing four times through a ball-bearing cell cracker (after W. Balch, Scripps Institute, La Jolla, California) followed by pelleting to 1 h at 100,000g in a Beckman TLA-45 rotor. Microsomes were then subjected to carbonate washing 21 or proteinase K digestion. Proteinase K digestion was for 60 min at 25 °C with 250 μ g proteinase K ml⁻¹ in 25 mM TrisHCl buffer (pH 7.5) containing 10 mM CaCl₂, 1 mM MgCl₂, 1 mM dibucaine, 100 mM NaCl with or without 1% Triton X-100. Digestion was stopped by the addition of 10 mM PMSF, precipitated with an equal volume of trichloroacetic acid and dissolved in reducing sample buffer before electrophoresis and transfer to nitrocellulose. The 9D6 anti-idiotypic secreting hybridoma cells were lysed in LB, immuno-precipitated with goat anti-mouse IgG followed by fixed *Staph. A* (Zysorbin; Zymed Inc.), washed four times in PBS containing an additional 0.3 M sodium chloride and 1% (v/v) Nonidet P-40, then washed once in water and eluted into reducing sample buffer before electrophoresis and transfer to nitrocellulose.

the retention of KDEL proteins could be overcome. Either the KDEL ligands could be coordinately upregulated to maintain functional levels in the lumen of the ER in the face of increased leakage, or the receptor itself could be upregulated to titrate the effect of the inhibiting antibody.

Analysis using a fluorescence-activated cell sorter (FACS) revealed that the anti-idiotypic hybridomas exhibited the same steady-state levels of PDI, BiP and GRP 94 as hybridomas producing a variety of irrelevant immunoglobulins (shown for PDI in Fig. 5a-c). Pulse-chase analysis confirmed that the anti-idiotypic hybridoma cells do not secrete large quantities of these normally retained KDEL proteins (data not shown). These results imply that the anti-idiotypic cells did not overcome the retention defect by increased ligand expression. Instead, the hybridoma cells contained greatly elevated levels of the putative receptor. Both 5D3 and 9D6 cells contain the normal 72K membrane-associated protein and large quantities of a heterogenous soluble form (Fig. 2d). The soluble antigen-antibody complex was also found at high levels in media conditioned by the anti-idiotypic hybridoma cells (Fig. 2b). The antigen in this material must have passed through the entire Golgi apparatus as a marked M_r shift was observed after neuraminidase treatment (data not shown). It is important to emphasize that soluble receptor is produced only by the two grossly abnormal anti-idiotypic-secreting hybridoma cell lines. No other cell lines have been found that secrete material immunoreactive with the anti-idiotypic antibodies.

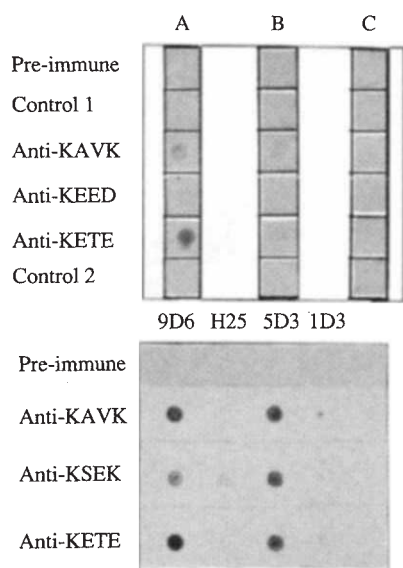


FIG. 3 The monoclonal anti-idiotypic antibodies recognize an idiope in rabbit anti-KDEL antisera. Upper panel: 5D3 antibody (lane A) specifically recognizes rabbit antisera to KDEL peptides, but not other rabbit antisera. This recognition is not seen with other IgM monoclonal antibodies (lane B), nor with second antibody alone (lane C). Lower panel: Rabbit anti-KDEL antisera recognize both 5D3 and 9D6 immunoglobulin, but do not react with other murine monoclonal antibodies (H25 and 1D3). This effect is not seen with rabbit antisera to other antigens.

METHODS. Polyclonal rabbit antisera to the peptides in Table 1 were prepared³⁵ and concentrated by ammonium sulphate precipitation. For the upper panel, aliquots of concentrated rabbit antisera (1 μ l) were dried onto nitrocellulose filters which were western blotted with culture supernatant of 5D3, culture supernatant from a control IgM secreting hybridoma, or blocking buffer and processed as described in Fig. 2. For the lower panel, hybridoma culture supernatant from 5D3 or 9D6 or control hybridomas was spotted onto nitrocellulose and probed with rabbit preimmune serum or antisera to KDEL using the same blotting protocol. In each case, the control antisera (Control 1 and Control 2) to irrelevant peptides had titres on their cognate antigens comparable to those of the anti-KDEL antisera on the immunizing peptides.

The poor growth of the anti-idiotypic-secreting hybridoma cells suggested that they might be under stress and prompted us to ask whether any of the observed up-regulation of antigen expression could be attributed to a stress response. In particular, as several of the soluble ER resident proteins are induced by glucose starvation, we tested this treatment for its ability to increase expression of the receptor. Figure 5d shows that the putative receptor was not upregulated under conditions that elicit strong induction of known glucose-regulated proteins. Furthermore, the putative receptor was not induced by treatment for up to 4 hours with the glycosylation inhibitor, tunicamycin, or the calcium ionophore, A23187 (Fig. 5d).

Soluble antigen binds to KDEL peptides and PDI

The soluble forms of the putative receptor, isolated from the medium of overexpressing hybridoma cells, were first tested for binding to KDEL-peptide affinity columns. The isolated receptor

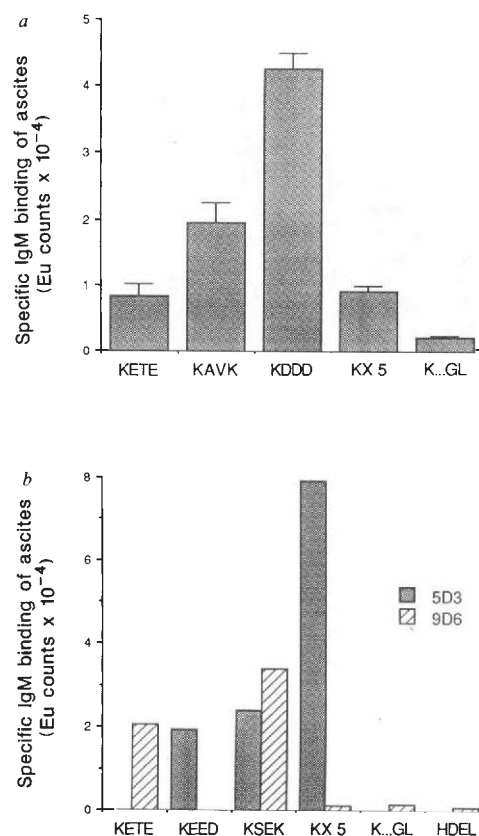


FIG. 4 Third-round immunizations generate anti-KDEL specific reactivity. a, Anti-5D3 antisera specifically recognize KDEL-terminated peptides. b, Spontaneous IgM responses in mice bearing anti-idiotypic hybridoma tumours also show anti-KDEL specificity.

METHODS. a, BALB/c mice were immunized four times, intraperitoneally, with paraformaldehyde fixed 5D3 hybridoma cells ($\sim 5 \times 10^6$ cells per mouse) at intervals of three weeks and bled 10 days after the final immunization. Sera were diluted 1:500 in DELFIA blocking buffer and assayed in triplicate on KDEL and K...GL peptide substrates by a solid-phase time resolved lanthanide fluorescence assay³⁶. Background values obtained with irrelevant mouse antisera were subtracted to give specific binding. The assay was performed three times using sera from three separate immunized animals and a representative set of results is shown. b, Ascites from unirradiated BALB/c mice carrying either 5D3 or 9D6 hybridoma tumours were clarified by ultracentrifugation and used at dilutions of 1:100 in a solid-phase DELFIA assay in triplicate on KDEL, K...GL and HDEL peptides. A highly purified affinity-isolated μ -chain-specific goat anti-mouse IgM second antibody conjugated to a Europium chelate was used as detecting antibody³⁶. This antibody has negligible cross-reactivity with mouse IgG. Although both antibodies were tested on all peptides, values below 2,000 Eu counts (photon counts of Europium fluorescence) are not visible in this representation.

bound specifically to the peptides containing the KDEL retention signal, but not to K...GL (Fig. 6a). The observed efficiency of binding was low (typically 3–5% of input counts), presumably because this isolated material differs from the 72K transmembrane receptor in normal cells; it is a soluble fragment, glycosylated by passage through the Golgi apparatus and associated with the anti-idiotypic antibody. Specific binding is obtained with material isolated from either anti-idiotypic hybridoma (Fig. 6a) and is divalent cation-dependent and maximal at physiological pH (data not shown). This indicates that divalent cations may play a part in switching the receptor between high- and low-affinity forms. The affinity of the unoccupied binding sites in the isolated antigen for KDEL ligands was estimated by a complementary experiment. This experiment measures the binding of radio-iodinated PDI to isolated antigen attached to Sepharose beads (Fig. 6b). Analysis of these data yields a dissociation constant K_d of 23 μ M. This binding can be competed by incubation with KX₅ peptide at a concentration of 100 μ M, a value similar to that calculated for KDEL tails in the ER lumen.

Localization of anti-idiotypic reactivity

Immunofluorescence with 5D3 on BHK-21 cells maintained at 37 °C (Fig. 7a) yielded a weak punctate staining pattern throughout the cytoplasm, which tended to concentrate in a crescent next to the nucleus. We reasoned that the low level of signal might reflect the fact that the receptor is almost continuously occupied during the normal functioning of the secretory pathway.

Treatments that interfere with the secretory pathway might be expected to interfere with recycling to the ER and result in decreased receptor-occupancy and therefore increased reactivity with the anti-idiotypic antibodies. Incubation at 15 °C blocks transport between ER and Golgi and causes elaboration of a novel tubular structure in the peri-Golgi region¹⁴. Incubation

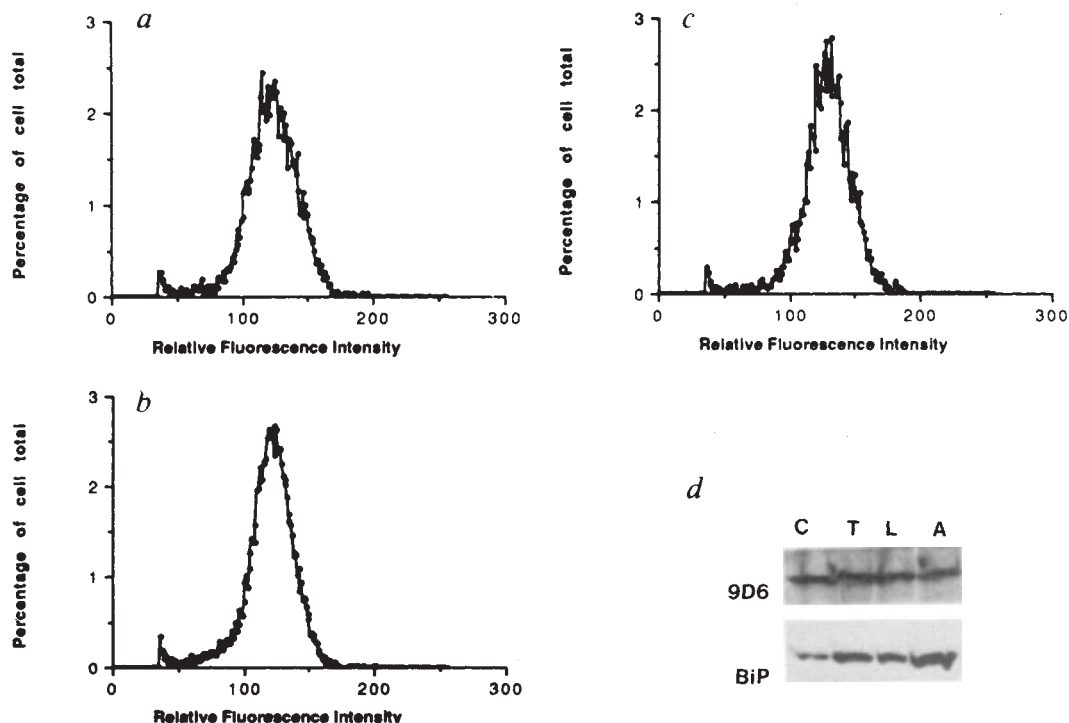
of BHK-21 cells at 15 °C for 2 hours caused an increase in the intensity of the immunofluorescence obtained after staining with 5D3, together with a tendency for the punctate stain to coalesce into larger strongly labelled regions in the cytoplasm (Fig. 7b). Under the same conditions, anti-KDEL antibodies gave their normal reticular pattern of immunofluorescence (data not shown). Hence, incubation at 15 °C, a treatment that interferes with ER to Golgi transport¹⁴, increases the accessibility of the 72K putative receptor to specific anti-idiotypic antibodies and alters its localization. This observation associates the receptor with the salvage compartment¹⁴.

Brefeldin A has a profound reversible effect on the exocytic pathway, which is manifest as a condensation of *cis* and medial Golgi into the ER^{24,25}. When NRK cells were treated with brefeldin A for 2 hours the normal Golgi structure was completely dispersed, although the reticular pattern associated with the ER labelling of rabbit anti-KDEL antisera remained essentially unchanged. Under these conditions staining with 5D3 revealed the usual vesicular pattern, but with increased intensity (Fig. 7c). After removal of the brefeldin A, 5D3 showed a reticular ER-like pattern of labelling (Fig. 7d) before returning to the normal staining pattern seen in Fig. 7a. This behaviour also associates the antigen with the salvage compartment²⁵.

Polyclonal antisera, raised in BALB/c mice by immunization with soluble receptor from either 5D3 or 9D6 cells, labelled the 72K band in VG cell lysates (Fig. 2d). These polyclonal antisera showed an immunofluorescent labelling pattern that was indistinguishable from the pattern obtained with 5D3. The widely distributed punctate labelling seen with these reagents showed the same tendency to concentrate to one side of the nucleus (Fig. 7e) and the same appearance in a juxtannuclear tubular compartment after a 15 °C block (data not shown) as observed with the anti-idiotypic antibody. Staining of the field shown in Fig. 7e with a polyclonal antiserum against the Golgi apparatus gives an overlapping but quite distinct cisternal

FIG. 5 a, b, c, PDI content of rapidly growing SP2/0 myeloma cells (a), 5D3 hybridoma cells (b) and VG hybridoma cells (c) is equal. d, Expression of the 5D3/9D6 antigen is not rapidly upregulated as part of the stress response.

METHODS. For a, b and c, cultures of SP2/0 myeloma cells and 5D3 and VG hybridoma cells in rapid growth were washed twice in PBS, fixed in suspension in methanol at –20 °C for 6 min, washed twice more in PBS and preincubated 1 h with PBS containing 10% (v/v) goat serum and 10 mM sodium azide. The fixed, permeabilized cells were then incubated with rabbit anti-PDI antiserum (Fig. 1) for 1 h, washed twice in PBS, reblocked with the goat serum buffer and labelled for 1 h with a rhodamine-conjugated affinity-purified goat anti-rabbit antibody³⁷. The cells were finally washed in PBS and analysed in an EPICS 4 (Coulter Electronics) FACS. Profiles from 50,000 cells, taken from a representative experiment are shown. For d, Vero cells were grown in normal growth medium (lane C), medium containing 50 ng ml^{–1} tunicamycin (lane T), glucose-free medium (lane L), or medium containing 0.5 μ g ml^{–1} A23187 (lane A) for 4 h. Cells were then collected, lysed, and equal aliquots analysed by SDS-PAGE and western blotted with



9D6 and a rabbit anti-BiP antiserum (rabbit anti-KEED). The western blots were repeated at several loadings of sample to ensure that neither second antibody nor substrate were limiting. The 9D6 immunoblot shown contains five times the protein-loading of the anti-BiP immunoblot.

pattern (Fig. 7f). Double-label immunofluorescence with the polyclonal antiserum to soluble receptor fragment (Fig. 7g) and 1D3 (Fig. 7h) show that the receptor and its ligand have overlapping but distinct locations.

Discussion

The anti-idiotypic approach to the identification of a receptor for the KDEL retention signal rests on the assumption that a single receptor is responsible for the recognition of the several KDEL proteins. Consistent with this assumption, three independent approaches led to an anti-idiotypic response against KDEL peptide antibodies and these independently-produced anti-idiotypic antibodies all recognized the same 72K transmembrane protein. The third round of immunization provided the strongest support for our initial assumption that one receptor serves several KDEL-terminated ligands. Antisera produced in response to immunization with 5D3 cells show a broad specificity for the KDEL signal but do not recognize the nonfunctional K...GL tail. A similar effect can be seen in the third-round response generated by production of ascites in non-irradiated mice from either of the anti-idiotypic hybridomas. These responses again show reactivity with several KDEL tail sequences but not with K...GL or HDEL peptides. This reactivity justifies the identification of the 72K antigen as a KDEL signal-binding protein and our localization and functional studies are consistent with a role in recognition of the KDEL retention signal *in vivo*. Henceforth we will refer to the antigen as the receptor.

Both 5D3 and 9D6 hybridomas secrete in large amounts a soluble form of the receptor that is highly glycosylated, oligomeric and complexed with antibody. Labelling experiments show that the production of soluble receptor exceeds antibody production in both hybridomas. Polyclonal antibodies against the secreted soluble receptor from 9D6 and 5D3 cells recognize the normal 72K receptor in VG cells. Therefore the secreted forms share antigenic determinants with the normal receptor, beyond those recognized by 5D3 and 9D6 antibodies. Whether the soluble form results from changes in transcriptional or post-transcriptional events remains to be seen, but we interpret overproduction of soluble receptor as a mechanism to titrate the anti-receptor antibodies in the lumen of the ER. Intact membrane-bound receptor present in the hybridoma cells and protected in this way from the antibody can then function sufficiently to permit survival of the cells.

The epitope recognized by 5D3 and 9D6 seems to be widely conserved throughout evolution. All of the vertebrate cell lines and tissues tested that show reactivity with our anti-tail antibodies (S.F., J.T. and D.V., manuscript in preparation) also react with the anti-idiotypic antibodies. None of the other cell lines tested showed the overexpression and secretion of the antigen observed in the 5D3 and 9D6 hybridomas. In normal cells the receptor seems to be a minor membrane protein. Our anti-KDEL tail antibodies show negligible reactivity with a peptide bearing the *S. cerevisiae* signal (HDEL) or with cell lysates from this yeast. Correspondingly, our anti-idiotypic antibodies show no significant reactivity in this organism.

Preliminary studies (results not shown) provide some intriguing insights about the receptor. Western blotting with a battery of our anti-KDEL tail antibodies indicate that the receptor is not itself a KDEL protein. The receptor is an intrinsic membrane protein that spans the membrane at least once (we cannot exclude several membrane-spanning domains). Nonreduced gels of VG lysates indicate the protein may be oligomeric and the antigen precipitated from 9D6 or 5D3 cells is disulphide-crosslinked to form apparent trimers and higher-order oligomers. Finally, the secreted receptor is post-translationally modified. That the secreted antigens from 5D3 and 9D6 cells, although cross-reactive, migrate at different M_r s reflects their sialylation, as this difference in mobility is abolished by neuraminidase-digestion. The receptor in normal cells does not

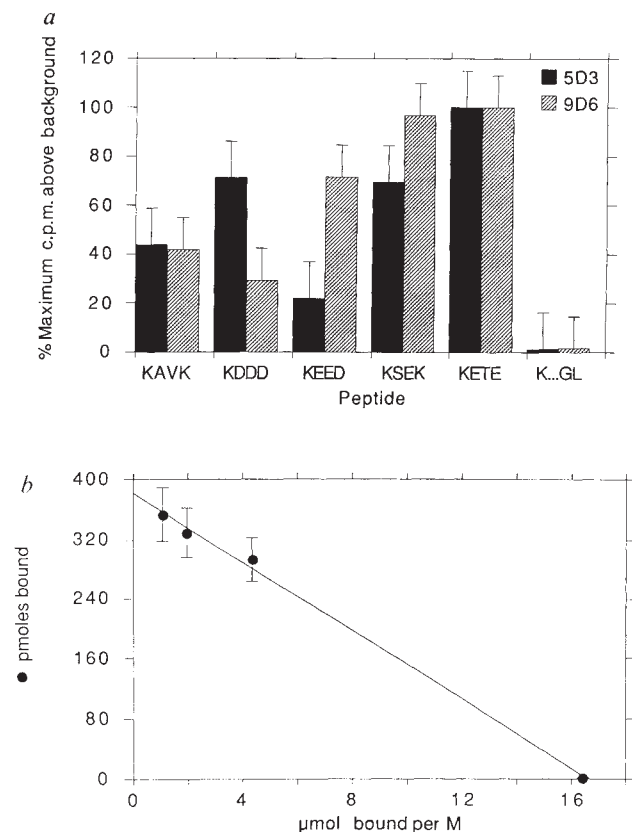


FIG. 6 Binding of isolated receptor to KDEL peptides and PDI. *a*, Radiolabelled soluble receptor isolated from either anti-idiotypic hybridoma cell line binds to several KDEL peptides, but not to the K...GL peptide. *b*, Iodinated PDI binds specifically to soluble antigen immobilized on Sepharose beads.

METHODS. 5D3 or 9D6 cells were labelled for 14 h with [35 S]methionine and the soluble fragment of the receptor isolated from the supernatant by chromatography on concanavalin A-Sepharose (Pharmacia) according to manufacturer's instructions, followed by dialysis against TN containing 1 mM dithiothreitol. *a*, Peptides were crosslinked to CnBr-Sepharose (Pharmacia) according to manufacturer's instructions. The peptide-Sepharose particles (50 μ l of a 1:1 v/v slurry) were pre-blocked by incubation at room temperature in 1 ml blocking buffer (TN 2% Tween-20, 1 mM CaCl₂, 1 mM MgCl₂, 20% FCS) for 2 h with gentle agitation, then incubated with constant aliquots of the labelled receptor overnight at room temperature. The peptide-Sepharose was then washed three times in blocking buffer without FCS and counted in a liquid scintillation spectrometer. The results shown are the average of four experiments, with each point in triplicate. Error bars indicate the standard error. Background corresponding to beads alone has been subtracted. In a typical experiment, the background value was 650 counts and the highest sample value was 1,800 counts or 3–5% of input counts. All values except K...GL and KEED (5D3) are significantly above background ($P < 0.05$). Gel electrophoresis of a larger quantity of KSEK-Sepharose after incubation with isolated 5D3 receptor showed that the only radio labelled protein bound co-migrated with the soluble form of the receptor. *b*, PDI was isolated from bovine liver by the method of Lambert and Freedman³² as modified in Tooze *et al.*²¹. PDI was iodinated using Iodobeads (Pierce) according to manufacturer's instructions at a concentration of 5 μ g ml⁻¹ and separated from free label by chromatography on Sepharose G-50. The specific activity of the labelled PDI was 1.05×10^7 c.p.m. μ g⁻¹ (0.18 atoms iodine per PDI tail). 9D6 soluble antigen was isolated as above and coupled to cyanogen bromide-activated Sepharose 4B according to manufacturer's instructions at a concentration of ~5 mg per ml of beads. For the binding assay, beads were pre-blocked by incubation with Blotto containing 1 mM DTT 1 mM CaCl₂ and 1 mM MgCl₂ for more than 1 h at room temperature and then incubated 1 h at room temperature with 35,000 c.p.m. ml⁻¹ of labelled PDI in the same buffer, together with unlabelled PDI at concentrations varying between 0 and 3 mM. Beads were then pelleted and the percentage of free counts calculated. Results of a representative experiment carried out in triplicate are shown as means and standard deviation. The line indicates a K_d of 23 μ M, and has a correlation coefficient of 0.9986. In four independent experiments K_d s of 20–50 μ M were found. Control experiments with beads alone showed a K_d greater than 10 mM.

show this high degree of glycosylation, presumably because it is not exposed to the later stages of the secretory pathway.

The localization of the receptor is central to an understanding of its function in the cell. The patterns of immunofluorescence-labelling of NRK cells at 37 °C with 5D3, which should be specific for receptor that does not have KDEL ligand bound to it, indicate that the receptor is in a compartment distinct from the ER and the Golgi apparatus but related to them both. The labelling of cells incubated at 15 °C establishes that the receptor is in the 15 °C compartment, which we equate with the salvage compartment. The patterns of labelling during brefeldin A treatment and recovery from it are also consistent with the proposal that unoccupied receptor is in a compartment between the ER and the Golgi stack, that is, the salvage compartment. Here, the unoccupied receptor is positioned appropriately to capture KDEL proteins that escape from the ER and to recycle them. Results obtained with polyclonal and monoclonal antibodies against isolated receptor show a very similar labelling pattern, suggesting that only low levels of receptor are ever found in other cellular compartments. We intend to investigate the localization of the receptor by EM immunocytochemistry.

The binding of a recycling receptor to KDEL tails must be modulatable. The receptor must have a high affinity for KDEL tails when it captures them and a lower affinity under the conditions of the ER where they are released. The secretion of a soluble form by the hybridomas allowed us to isolate receptor for binding studies. Binding of this material to peptide columns demonstrated that it specifically recognized the KDEL terminated peptides rather than a K...GL peptide. This specific binding was strongest near physiological pH and was divalent cation-dependent. Pure labelled PDI bound to isolated receptor

with a K_d of $\sim 23 \mu\text{M}$. Although post-translational modifications of the secreted receptor may cause a change in the characteristics of binding, this K_d is within the physiological range. The concentration of KDEL tails calculated for the ER lumen is well above this constant, indicating that our binding protein is a plausible candidate for the physiological receptor. In addition, this value suggests that the difference between the high-affinity (salvage compartment) and low-affinity (ER) forms need not be large. The transferrin receptor, for example, has only a 20-fold greater affinity for diferritransferrin than for apotransferrin²⁶. Such a change in the affinity of our putative receptor would allow it to function in the recycling and release of KDEL proteins into the ER lumen, where their concentration is high. The size of this affinity constant also provides an explanation for the difficulty experienced by us, and others, in isolating a KDEL receptor by conventional affinity methods. Finally, the conclusion that receptor binding is divalent cation-dependent is supported by recent work²⁷ that suggests the involvement of Ca^{2+} in the retention of KDEL-terminated proteins in the ER *in vivo*. Furthermore, we have observed that the availability of cellular receptor for binding to anti-idiotypic antibody in immunofluorescence experiments decreased after incubation of cells in medium containing the calcium ionophore A23187 and Ca^{2+} (S.F., J.T. and D.V., manuscript in preparation).

This work has identified a KDEL binding protein that we think is a good candidate for the physiological receptor. In summary, our evidence that the 72K protein is indeed the receptor comprises the following: (1) separate routes beginning with different KDEL sequences identify the same oligomeric transmembrane antigen; (2) a broadened specificity is observed in the third-round antibodies; (3) the antigen is concentrated

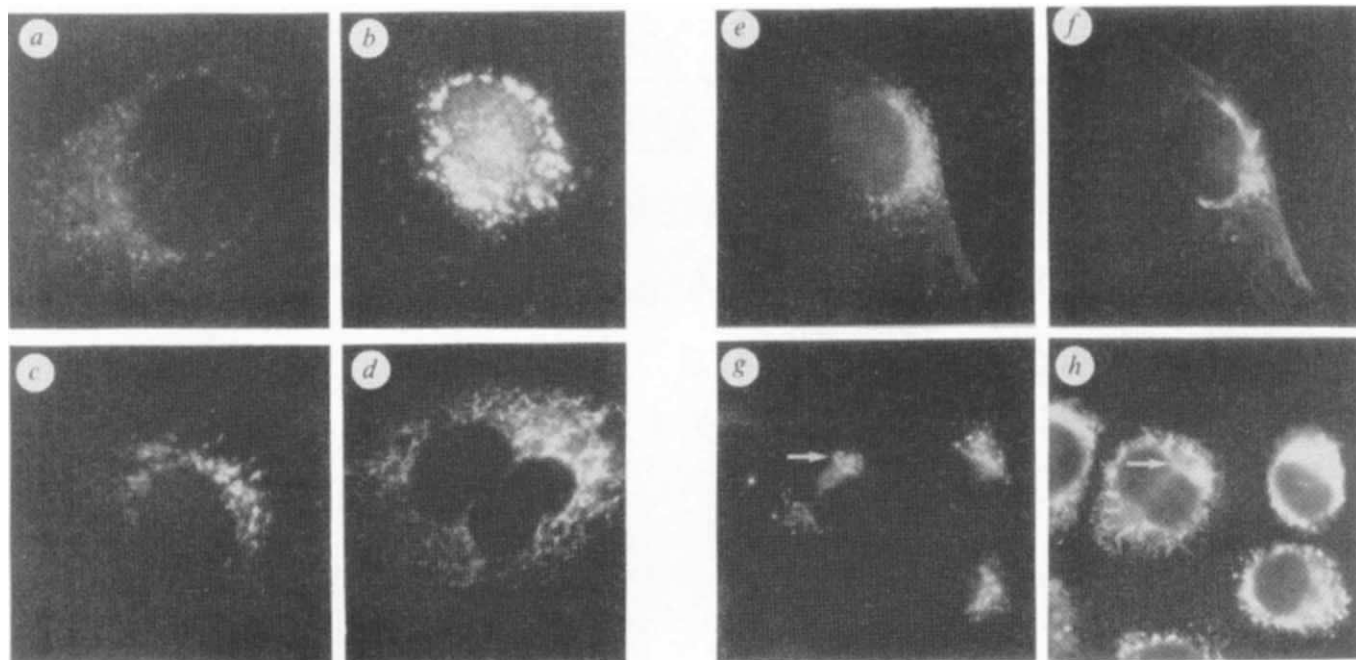


FIG. 7 Intracellular location of the receptor. Methanol-fixed NRK cells were labelled with 5D3 culture supernatant after incubation at 37 °C (a), 2 h at 15 °C (b), 2 h at 37 °C in the presence of $10 \mu\text{g ml}^{-1}$ brefeldin A (c), after 30 min recovery at 37 °C from a 2 h brefeldin A block (d). e, Labelling of NRK cells with mouse polyclonal anti-receptor antiserum. f, The same cell labelled with a rabbit polyclonal antiserum against the Golgi apparatus. g, Labelling of methanol-fixed untreated NRK cells with an affinity-purified rabbit polyclonal antiserum to the soluble receptor fragment. The same field is shown in (h) double-labelled with the mouse monoclonal antibody, 1D3, to reveal the distribution of PDI. The arrow indicates a region of a flattened cell in which receptor is concentrated in an area from which PDI is excluded. METHODS. For immunofluorescence NRK cells were grown on multiwell

slides, treated as described above, washed briefly in PBS of the appropriate temperature and fixed in methanol at $-20 \text{ }^{\circ}\text{C}$ for 6 min. The cells were washed twice in PBS and incubated in blocking buffer (PBS containing 10% (v/v) normal goat serum) at room temperature for 30 min in a humidified chamber. First antibody incubations were for 1 h at room temperature. The slides were washed five times with PBS, re-blocked with blocking buffer then exposed to fluorochrome-conjugated second antibody (Zymed Inc.) diluted according to the manufacturer's instructions in blocking buffer for 1 h. After extensive washing in PBS, the slides were mounted with Moviol and examined in a Leitz Axiophot microscope. Photography was with Tri-X rated at 1,600 ASA. Final magnification, $\times 6,300$ (all panels).

in a compartment intermediate between ER and Golgi; (4) the KDEL binding site lies in the lumen of the secretory pathway; (5) the anti-idiotypic hybridomas dramatically upregulate this protein; (6) isolated antigen binds specifically to peptides containing the retention signal and (7) isolated antigen binds physiological ligand with a K_d compatible with a recycling model for ER retention. Although it remains formally possible that the localization and binding properties of this protein are only coincidentally those expected of a KDEL receptor, the properties of this protein are consistent with the model of Munro and Pelham¹⁰, and indicate additional features of ER retention. The binding site of one receptor species can accommodate all known KDEL signals, hence several receptors are not necessary. The receptor is a minor transmembrane protein, which must therefore operate by a recycling mechanism. The receptor is not normally detectable within the bulk of the ER or the Golgi

apparatus. The receptor appears restricted to a relatively small region that may represent the exit sites from the ER and the return sites for captured ligands. Both 15 °C incubation and brefeldin A treatment cause changes in receptor localization consistent with it being predominantly present in the intermediate or salvage compartment. The observed K_d of receptor for ligand would allow the receptor to capture ligand in the salvage compartment and release it into the ER without requiring large changes in affinity constant. The moderate changes necessary could be mediated by an environmental factor such as pH or divalent cation concentration. Whether the receptor recycles continuously or only when occupied by ligand is unclear; but we note that not only is the receptor a transmembrane protein but it and several of its ligands are oligomeric³. This opens the possibility that the receptor signals its occupancy to the cytoplasm by clustering²⁸. □

Received 22 December 1989; accepted 27 April 1990.

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ACKNOWLEDGEMENTS. We acknowledge the technical assistance and forbearance of P. Buck and M. Kail. We thank our colleagues R. Frank and H. Gausepohl for the synthesis of the peptides and for useful advice on peptide chemistry. We thank B. Burke for the gift of a polyclonal antiserum against a protein of the Golgi apparatus. We also thank P. Walter and H. Pelham for their comments and encouragement.

Major determinants of the specificity of interaction between small nuclear ribonucleoproteins U1A and U2B'' and their cognate RNAs

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The basis of the specificity of interaction of U1 and U2 small nuclear (sn)RNAs and their cognate binding proteins, U1A and U2B'', has been examined. The U1A protein recognizes U1 snRNA on its own, whereas U2B'' binds specifically to U2 snRNA only in the presence of a second protein, U2A'. Exchange of two nucleotides between the two RNAs or of eight amino acids between the two proteins reverses binding specificity.

LITTLE information on the structural basis of RNA-protein binding is available. Analysis of common structural motifs in families of proteins that bind to other ligands, for example nucleotide cofactors or DNA, has been useful in the study of

these proteins. We have therefore examined the role in specific RNA binding of a conserved motif found in ribonucleoproteins (RNPs). The RNP motif is of 70–90 amino acids^{1–3}. The most conserved portion is an eight-amino-acid stretch called the RNP consensus sequence⁴ (RNP-CS; also RNP1 (ref. 5) or the octamer region⁶). Several protein components of the U snRNP group of nuclear ribonucleoproteins contain RNP motifs. These include U1 70K (refs 3, 7, 8), U1A (ref. 9) and U2B'' (ref. 10). Previous work with U1 70K and U1A identified minimal segments of these two proteins that were required for binding to U1 RNA (refs 6, 11). The minimal segments contained a copy of the RNP motif but, in both cases, flanking sequences were also required for specific RNA binding. This left open the question of whether binding specificity is due to sequences in the motif or to the unique sequences outside it.

To approach this problem we have examined the interaction between two proteins, U1A and U2B'', and their cognate RNAs,

U1 and U2 snRNA. The U1A and U2B' proteins share extensive sequence similarity⁹. The C-terminal 77 amino acids of the two proteins are 86% identical, where residues 7–101 of U1A and 4–98 of U2B' are 77% identical. These two segments are separated by dissimilar regions. Each protein contains two copies of the RNP motif⁶, which lie between amino acids 12–84 and 210–277 in U1A and 9–81 and 153–220 in U2B'. In U1A the C-terminal RNP motif is not required for specific RNA binding¹¹.

Specific U2B' binding to RNA requires U2A'

The U1A protein binds specifically to U1 RNA *in vitro* in the absence of other U snRNP components^{11,12}. To test whether U2B' binding to U2 RNA was comparable, human U2B' protein was synthesized *in vitro* and its binding to *Xenopus* U2 snRNA was tested and compared with nonspecific binding to U6 snRNA. U2B' bound to RNA, but nonspecifically (Fig. 1, lanes 4, 5). Because U2B' is associated with U2 snRNA *in vivo*, some component required for specific interaction might have been missing from the assay system. To investigate this possibility, U2B' protein made *in vitro* was complemented with HeLa cell nuclear extract and its association with U2 and U6 reassessed. In the presence of nuclear extract the nonspecific binding of U2B' to U6 was reduced and U2-specific binding was observed (Fig. 1, lanes 10 and 11). Earlier studies *in vivo* suggested that U2B' is closely associated with U2A' in the U2 snRNP (refs 13–15). The effect of *in vitro* synthesized U2A' protein¹⁶ on RNA binding was therefore tested. Radiolabelled U2A' made in wheat-germ extract (Fig. 1, lane 1) did not bind detectably to RNA (Fig. 1, lanes 2, 3). When wheat-germ extract programmed with U2A' messenger RNA (in the absence of [³⁵S]methionine) was added to U2B', specific binding of U2B' to U2 RNA was observed (Fig. 1, lanes 6, 7). Addition of control wheat-germ extract had only a slight effect on the specificity of U2B' binding (Fig. 1, lanes 8, 9). Thus, specific association of U2B' with U2 snRNA required U2A'. This will be discussed in more detail below.

Two nucleotides crucial for binding specificity

Xenopus U2 snRNA can be divided into four stem-loops (I–IV) and two single-stranded regions (Fig. 2a; ref. 17, but see ref.

TABLE 1 Sequences and binding properties of U1 loop II and U2 loop IV mutants

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Sequences of the mutated loops are shown between the corresponding wild-type (wt) loop sequences. Mutated nucleotides are underlined and deleted nucleotides are shown by an underlined gap. An intermediate level of binding is indicated by –/+. The results of binding experiments with either the U1A or U2B' protein are indicated. The asterisks mark nucleotide positions crucial for discrimination between U1A and U2B' binding.

18). Previous *in vivo* studies^{13–15} identified hairpins III and IV as likely sites of interaction with U2B' and U2A'. Various deletion derivatives of U2 were prepared to define the requirements for U2B' binding *in vitro*. The U2 fragments were named according to which loops they retained (such as I and II, III and IV) or which had been deleted (for example, ΔIII). Deletion of hairpin IV abolished U2B' binding (Fig. 2b, lanes 4, 6), whereas deletion of other parts of the molecule had no effect. An RNA transcript containing only hairpin IV showed full

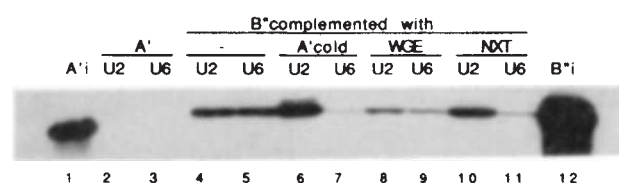


FIG. 1 U2B' requires U2A' for specific binding to U2 snRNA. Binding of *in vitro* translated U2A' (lane 1) and U2B' (lane 12) proteins to U2 and U6 RNAs was analysed. Although neither protein alone (lanes 2, 3, 4 and 5) or after the addition of control wheat-germ extract (WGE; lanes 8, 9) exhibited U2-specific binding, complementation of U2B' with either 10 μg HeLa cell nuclear extract (NXT; lanes 10, 11) or wheat-germ extract programmed with U2A' mRNA (lanes 6, 7) led to specific U2B'–U2 RNA binding.

METHODS. *In vitro* transcription and translation and protein–RNA binding assays have been described¹¹. Plasmids carrying U2B' or U2A' cDNAs were linearized and, together with T7 RNA polymerase, used for the synthesis of the corresponding messenger RNAs. The mRNAs were translated in wheat-germ extract in the presence of [³⁵S]methionine, resulting in the production of labelled U2B' or U2A'. U2B' protein, for unknown reasons, always appears as a doublet of relative molecular mass 28,000 (M_r , 28K). U2A' protein has a predicted M_r of 33K but migrates as 27K. Labelled U2A' or U2B' complemented with the components described above was mixed with the biotinylated U snRNA test substrate. After incubation, proteins bound to the RNA were precipitated with streptavidin coupled to agarose beads. After several washes, the bead pellet was resuspended in electrophoresis sample buffer and boiled to release the RNA-bound proteins. The supernatant was fractionated by SDS-PAGE, fluorographed, dried and autoradiographed. HeLa cell nuclear extract was made as described³¹.

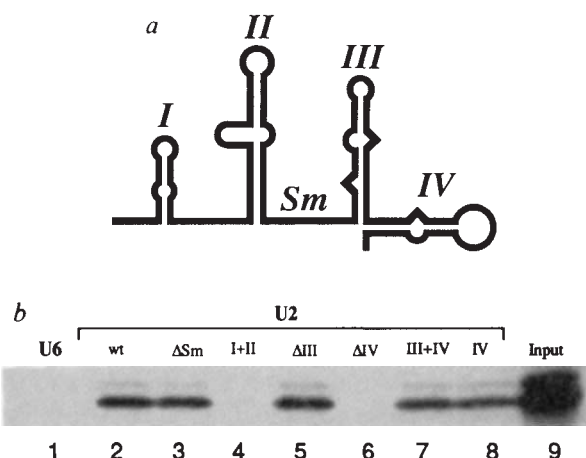


FIG. 2 Characterization of the *in vitro* binding site for U2B' protein. a, Possible secondary structure of *Xenopus* U2 (ref. 17) showing the four hairpin loop structures and the Sm binding site, which is necessary for interaction with the common U snRNP proteins¹³. b, Various U2 structural mutants were tested for their ability to bind U2B' protein. U6 snRNA (lane U6) was used as a negative control and wild-type U2 snRNA (lane wt) was the positive control. Mutant ΔSm has a modified version of the Sm binding site (A⁹⁹UUUUUG¹⁰⁵ → A⁹⁹UGAGUG¹⁰⁵). I+II lacks the third and fourth hairpins. ΔIII lacks the third hairpin, ΔIV lacks the fourth hairpin, III+IV consists of the third and fourth hairpins, and IV consists of the fourth hairpin alone. The amount of U2B' protein used for each binding assay is shown in the input lane.

METHODS. A T7 RNA polymerase promoter was introduced upstream of the coding part of a *Xenopus* U2 snRNA gene^{20,31}. This construct was subcloned into a pEMBL plasmid derivative, pM9 (P. Monaci and A. Nicosia, unpublished results) allowing the production of single-stranded DNA when cells carrying this plasmid are infected with the helper phage R408. The single-stranded DNA was mutated by site-directed mutagenesis³⁰.

binding activity in this assay (Fig. 2b, lane 8). Thus, the conserved¹⁹ hairpin IV is the binding site for U2B'' *in vitro*.

The loop sequence of hairpin II of *Xenopus* U1 RNA is largely responsible for the specific binding of the U1A protein¹¹. Comparison with hairpin IV of *Xenopus* U2 RNA revealed that the loop regions of these two hairpin are extremely similar (Fig. 3a), whereas the stems are different^{20,21}. This, together with its conservation^{19,22}, suggested that the loop of U2 hairpin IV might be responsible for sequence-specific binding of U2B''.

To test this hypothesis, a series of point mutants in U1 hairpin II were made. They are listed in the upper part of Table 1. The sequence alterations in mutants 1.2, 1.3 and 1.4 result in the stepwise transformation of the U1 loop II sequence to that of U2 loop IV. Changing C71 to G (Fig. 3a; Table 1) in mutant 1.2 resulted in a low level of specific U2B'' binding (Fig. 3b, compare lane 5 with the negative control, lanes 2 and 4). Combining this mutation with the insertion of an A between U72 and C73, to generate mutant 1.3, resulted in U2B'' binding at a level comparable to that of U2 (Fig. 3b, lane 6). Addition of a U residue at each extremity of the loop sequence (mutant 1.4), had no further effect. The results are summarized in Table 1. Thus, alteration of only two nucleotide positions in U1 RNA enables it to bind specifically to U2B''.

An analogous series of U2 mutants were created by mutation of loop IV (Table 1, 2.1–2.4). Deletion of A168, to generate 2.2 (Fig. 3a; Table 1) also does not allow U1A binding (Fig. 3c, lane 4). But combination of this mutation with the alteration of G166 to C (mutant 2.3) allowed binding of U1A to a level

comparable to that of U1 (Fig. 3c, lanes 1, 3). Deletion of the two U residues at the 5' and 3' extremities of the loop (mutant 2.4) had no further effect (Fig. 3c, lane 6). The results are summarized in Table 1. Thus, alteration of two nucleotide positions in U2 RNA converted it to a substrate for U1A binding.

The region of U2B'' required for U2 RNA binding

To define which region of U2B'' was required to bind U2 RNA, truncated U2B'' derivatives were generated (Fig. 4, marked with white dots) and their RNA binding was tested in the presence of control, full-length U2B'' protein (Fig. 4, arrowhead). Binding to U1 RNA was used as a control of specificity. A fragment of U2B'' corresponding to the N-terminal 76 amino acids was unable to bind to RNA (Fig. 4, lanes 2–4). Addition of twelve or more amino acids produced proteins which displayed specific binding to U2 RNA (Fig. 4, lanes 5–19). This defines the minimal segment of U2B'' protein capable of binding specifically to U2 RNA as lying between amino acids 1–88. This region of the protein includes the N-terminal copy of the RNP motif^{6,9}. The C-terminal copy of the RNP motif (residues 153–220) and the U2B''-unique region (residues 101–147) are not required for specific RNA binding.

RNP motif residues and RNA binding specificity

That only two nucleotide positions in U1 and U2 snRNAs are required to distinguish them as substrates for U1A or U2B'' binding suggested that the protein determinants responsible for this specificity might also be relatively simple. To test this,

FIG. 3 Nucleotide determinants of U1A and U2B'' binding specificity. a, Schematic secondary structures of hairpin II of *Xenopus* U1 snRNA and hairpin IV of *Xenopus* U2 snRNA. Base-paired nucleotides of the stem are shown by a bar, whereas unpaired or bulged nucleotides are shown by dots. No stem sequence is conserved between the RNAs. The sequence of each loop is shown. Nucleotides specific for the *Xenopus* U1 or U2 loops are indicated by an arrow or arrowheads respectively and their positions in the RNAs are numbered. Nucleotides that determine specific binding to U1A or U2B'' are circled. Note that both loops contain the sequence AUUG-CANUXCC, where N denotes G or C and X denotes A or no nucleotide. b, *In vitro* binding of U2B'' to U1 loop II snRNA mutants. B''i (lane 1) is the amount of input protein used in each binding assay. Lanes 2–7 show the amount of U2B'' protein bound to the indicated RNA substrates. c, *In vitro* binding of U1A to U2 loop IV snRNA mutants. Lanes 1–6 show the amount of U1A protein bound to the indicated RNA substrates.

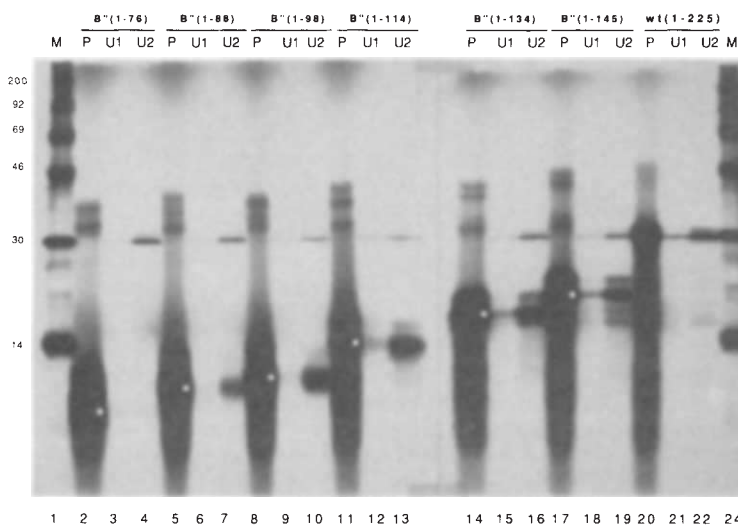
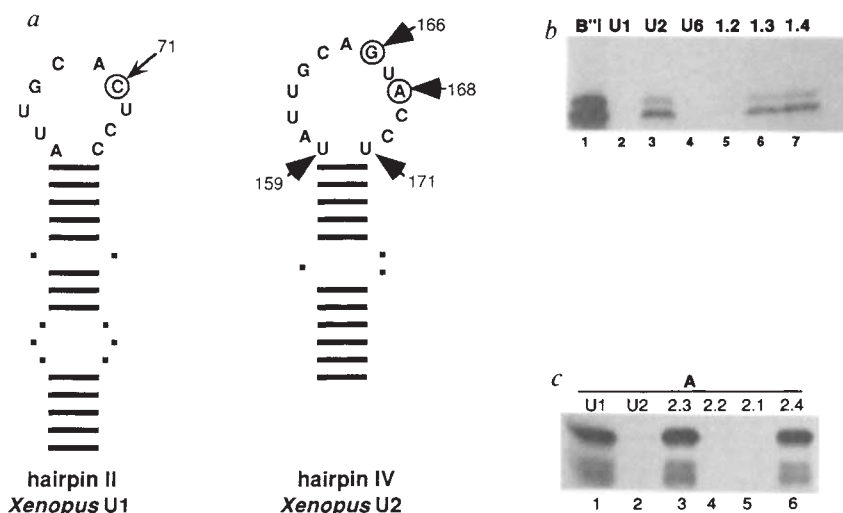


FIG. 4 *In vitro* binding of C-terminal truncated U2B'' derivatives. Binding capacity of C-terminal truncated U2B'' proteins (labelled with a white dot) was tested using the binding assay described in the legend to Fig. 1. U1 snRNA was the control for nonspecific RNA binding and wild-type (wt) U2B'' protein was included in each binding assay as an internal positive control (arrowhead). The molar ratio between wild-type U2B'' and the mutants was between 1:2 and 1:3. For each truncated protein the amount of protein used in the binding assay (P lanes), the amount of protein bound to U1 RNA (U1 lanes) and to U2 RNA (U2 lanes) is shown. M lanes contain M_r standards.

METHODS. Unique *Bam*HI restriction sites were introduced in a U2B'' cDNA at amino acid positions 77/78, 89/90, 99/100, 115/116, 135/136 or 146/147 by site-directed mutagenesis. The introduction of a *Bam*HI site leads to the substitution of glycine and serine for the original amino acids. The mutated cDNAs were linearized at the introduced *Bam*HI sites, transcribed *in vitro* by T7 RNA polymerase and the resulting mRNAs translated in wheat-germ extract in the presence of [³⁵S]methionine. The binding of the U2B'' derivatives was tested in the presence of HeLa nuclear extract.

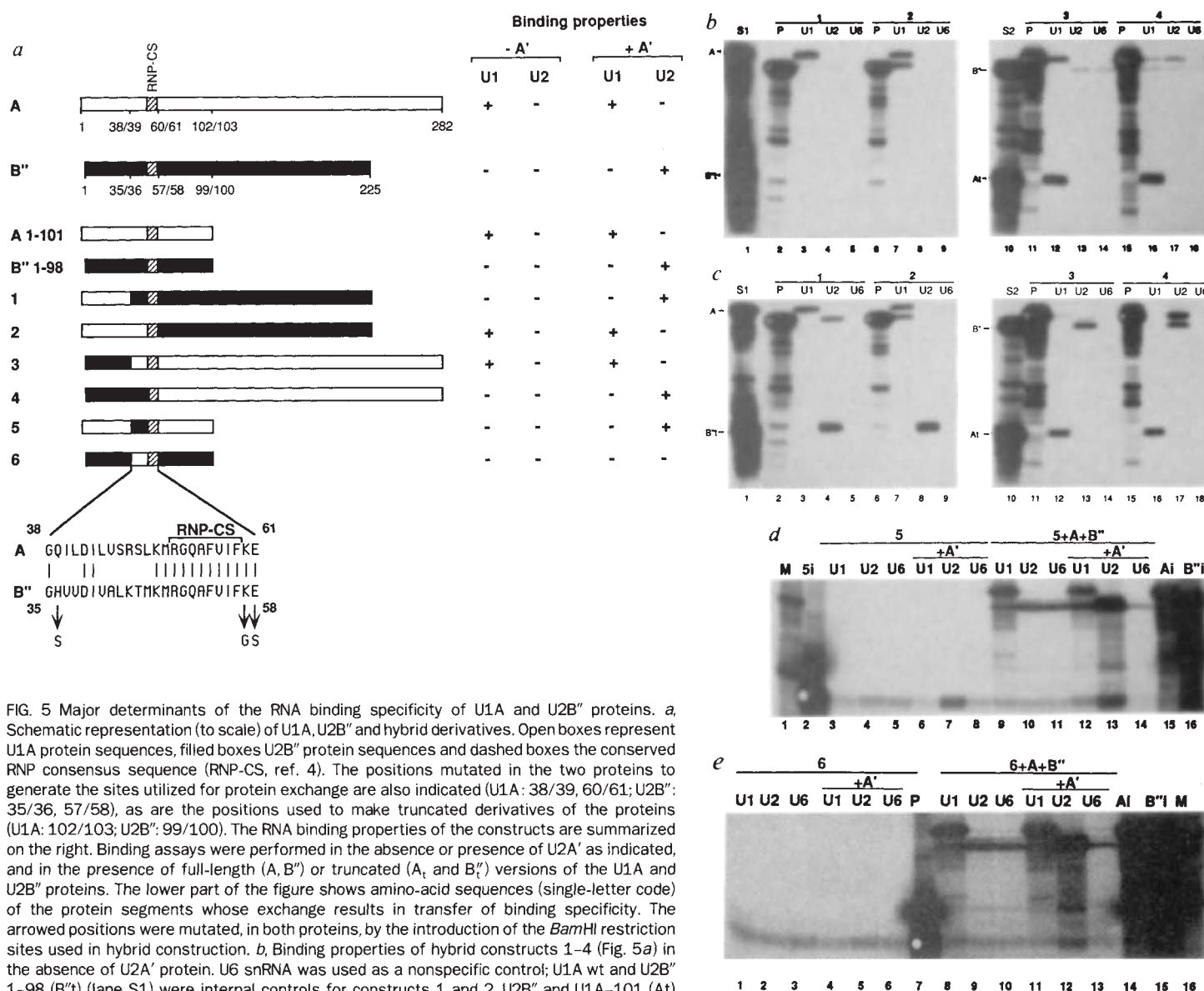


FIG. 5 Major determinants of the RNA binding specificity of U1A and U2B'' proteins. *a*, Schematic representation (to scale) of U1A, U2B'' and hybrid derivatives. Open boxes represent U1A protein sequences, filled boxes U2B'' protein sequences and dashed boxes the conserved RNP consensus sequence (RNP-CS, ref. 4). The positions mutated in the two proteins to generate the sites utilized for protein exchange are also indicated (U1A: 38/39, 60/61; U2B'': 35/36, 57/58), as are the positions used to make truncated derivatives of the proteins (U1A: 102/103; U2B'': 99/100). The RNA binding properties of the constructs are summarized on the right. Binding assays were performed in the absence or presence of U2A' as indicated, and in the presence of full-length (A, B'') or truncated (A_i and B''_i) versions of the U1A and U2B'' proteins. The lower part of the figure shows amino-acid sequences (single-letter code) of the protein segments whose exchange results in transfer of binding specificity. The arrowed positions were mutated, in both proteins, by the introduction of the *Bam*HI restriction sites used in hybrid construction. *b*, Binding properties of hybrid constructs 1–4 (Fig. 5a) in the absence of U2A' protein. U6 snRNA was used as a nonspecific control; U1A wt and U2B'' 1–98 (B''_i) (lane S1) were internal controls for constructs 1 and 2, U2B'' and U1A–101 (A_i) (lane S2) were internal controls for constructs 3 and 4. The positions of the internal protein controls are indicated on the left of lanes 1 and 10, whereas the positions of the mutant constructs (1–4) are indicated by white dots in the P lanes. P lanes represent the amount of chimaeric protein used in the binding assay. *c*, As *b*, except that each binding reaction was complemented with U2A' protein made *in vitro*. *d*, Binding properties of hybrid construct 5 either alone (lanes 3–8) or in the presence of U1A and U2B'' internal controls (lanes 9–14). The binding experiments were conducted in the absence (lanes 3–5 and 9–11) or in the presence (lanes 6–8 and 12–14) of U2A' protein. The hybrid protein is marked with a white dot in lane 2. Lanes 5i, Ai and B''i represent the amount of protein used in each binding assay. *e*, Same as *d*, but for hybrid construct 6.

METHODS. Unique *Bam*HI sites were introduced into U1A and U2B'' cDNAs. These sites, together with unique restriction sites in flanking regions, were used to exchange parts of the cDNAs to create the hybrid constructs 1–4. To obtain mutants 5 and 6, a second *Bam*HI site was introduced at amino-acid positions 60/61 into a U1A cDNA already carrying a *Bam*HI site at positions 38/39 and similarly at positions 35/36 and 57/58 of a U2B'' cDNA. The two double mutants were digested with *Bam*HI, the small internal *Bam*HI fragments were gel purified and ligated into the vector fragment obtained by digestion of the other cDNA. Unique *Bgl*II sites introduced into both constructs were used to generate proteins ending at position 101 (construct 5) or at position 98 (construct 6).

chimaeric proteins were constructed using restriction sites introduced into analogous positions of the complementary DNAs (Fig. 5a). Only sites whose introduction did not affect binding were used for hybrid construction. The ability of the hybrids to bind to RNA was tested in the presence of specificity controls in the absence (Fig. 5b) and presence (Fig. 5c) of the U2A' protein. Mutant 1 (Fig. 5a) showed the same binding specificity as U2B'', binding specifically to U2 RNA only in the presence of U2A' (Figs. 5b and c, lanes 2–5). The mutant protein is marked with a white dot. The internal controls are U1A and truncated U2B'' protein. By contrast, mutant 2 has the same binding specificity as U1A (Fig. 5b and c, lanes 6–9). In mutants 3 and 4, the converse regions of U2B'' and U1A protein were

exchanged (Fig. 5a). Mutant 3 behaves like U1A (Fig. 5b and c, lanes 11–14, note that the internal controls are now wild-type U2B'' and the truncated U1A protein). Mutant 4 behaves like U2B'' (Fig. 5b and c, lanes 15–18), although it can bind weakly to U1 and U2 in the absence of added U2A'.

These results define short regions of the two proteins whose exchange results in the transfer of RNA-binding specificity. The sequences of the relevant segments are shown in the lower part of Fig. 5a. Within these sequences, positions 39, 60 and 61 of U1A and 36, 57 and 58 of U2B'' cannot be required for RNA binding, because their alteration during the construction of the mutant proteins had no effect. Furthermore, only 8 of the other 21 amino acids exchanged differ in the two proteins. To further

define the importance of these eight amino acids in specific binding, mutants 5 and 6 were constructed. In these two mutants, short regions of U2A and U2B' were exchanged into truncated versions of the corresponding proteins (Fig. 5a).

Mutant 5 (Fig. 5d) behaved like U2B'. The protein (marked with a white dot) bound to U2 RNA only in the presence of U2A' protein (lanes 3–8). Mutant 6, however, did not show any specific RNA binding (Fig. 5e; the signal running just below mutant 6 in lane 12 is due to a truncated fragment of the U2B' internal control) even in a full-length version in which amino acids 99–225 of U2B' protein were present (data not shown). Nevertheless, the results with mutants 1–5 firmly implicate the eight amino-acid differences in the segments of U1A and U2B' shown in Fig. 5a as being crucial for RNA binding specificity. Furthermore, the results imply that the same portion of U2B' may be sufficient to allow interaction with U2A' protein because mutant 5 (Fig. 5a) requires U2A' for specific RNA binding. That the binding of mutants 3 and 4 to U1 RNA is reduced in the presence of U2A' (Fig. 5b and c) suggests that the N-terminal region of U2B' is also involved in U2A' binding. Direct studies of the interaction between these two proteins will be required to confirm these conclusions.

Discussion

We have examined determinants of specificity in the interaction between two U snRNP proteins, U1A and U2B', and their cognate RNAs. In this way, compact regions in the two proteins, and the two RNAs, which are main determinants of binding specificity and sufficient to explain the discrimination between U1 and U2 snRNA binding, have been identified. As the two proteins⁹ and the two RNA substrates (Fig. 3a) share extensive sequence similarity, it is to be expected that the sequences involved in U1–U2 discrimination are not the only residues required for overall binding specificity. Indeed, the possibility that other amino acids have a more minor role in U1–U2 discrimination has not been ruled out, and might explain the binding behaviour of mutants 4 and 6. We are now attempting to devise quantitative assays to allow measurement of the contribution of the regions involved in specificity determination to the total binding energy.

Perhaps the most surprising result about the binding of the U1A and U2B' proteins to RNA is that, despite their extensive similarity⁹, U1A can recognize RNA on its own, whereas U2B' requires a second protein, U2A', to specifically bind to U2 RNA. There are several simple possible schemes for the U2 interaction of U2B' and U2A'. U2A' could bind specifically to U2, with U2B' binding to the complex or to U2A' protein itself, or to an

altered conformation of U2 RNA which is induced by U2A' binding. Because U2A' does not bind to RNA in the *in vitro* assay (Fig. 1), we consider these possibilities unlikely. It is more probable that U2A' and U2B' interact either in solution, or after U2B' has bound nonspecifically to RNA, inducing a conformational change in U2B' which allows specific U2 snRNA binding. There are several strong reasons for believing that U2B' interacts directly with U2 RNA. First, the high nonspecific RNA binding of U2B' in the absence of U2A'. Second, the extensive similarity between the U1A and U2B' proteins⁹, and the similarity between their respective RNA binding sites which is described here. Third, it seems extremely unlikely that the hybrid protein constructs (1–4, Fig. 5a) would all bind to RNA if U2B' were not itself an RNA-binding protein.

Because the minimal segments of U1A and U2B' required for RNA binding both contain an RNP motif, a structural element present in many RNA binding proteins, it is possible that the results presented here can be generalized to other members of the family. The region of U1A and U2B' here defined as a main determinant of binding specificity lies just upstream of the RNP consensus sequence (Fig. 5a) in one of the most variable regions of the motif^{1,6,23}. This variability would be consistent with a general role for this region in the determination of binding specificity, but further evidence is required.

Among DNA binding proteins, exchange of one or a few amino acids between members of the helix–turn–helix or zinc-finger protein families can result in the exchange of DNA binding specificity^{24–28}. These results would support the hypothesis that proteins that contain similar DNA-binding motifs recognize DNA in a similar way. But this view seems to be an oversimplification as different helix–turn–helix proteins can bind to DNA in recognizably different ways (reviewed in ref. 29). There are additional reasons to doubt that RNP motif proteins will all recognize RNA in exactly the same way. The binding substrates of this family of proteins have great structural variability¹¹. It therefore seems probable that different members of the RNP motif family will use different protein structural elements, perhaps even outside the RNP motif, to achieve specificity. The region of U2B' that is required for interaction with the U2A' protein seems to correspond to all or part of the conserved N-terminal RNP motif. So the motif is not only involved in protein–RNA but also in protein–protein interactions. Because many members of the RNP motif family exist in complex RNPs in which protein–protein interactions are likely to occur, this may turn out to be a common function. □

Received 5 February; accepted 18 April 1990.

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ACKNOWLEDGEMENTS. We thank B. Dobberstein, J. Hamm, M. Hentze, A. Lamond, H. Parry, L. Philipson, D. Tollervey, P. Vankan and R. Zeller for comments on the manuscript. We thank the EMBL photolab, H. Seifert and I. Fraignaud for help in the preparation of the manuscript. This work was supported by EMBO (W.B. and D.S.). The work in Holland was carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with the financial aid of the Netherlands Organization for the Advancement of Pure Research (ZWO) and the Netherlands League Against Rheumatism.

Effect of the Great Attractor on the cosmic microwave background radiation

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ANISOTROPY in the cosmic microwave background radiation (CMB) is expected as a result of fluctuations in gravitational potential caused by large-scale structure in the Universe. The background radiation is redshifted as it climbs out of gravitational wells (the Sachs–Wolfe effect¹). Here we present a map of the anisotropy in CMB temperature $\Delta T/T$ of our region of the Universe as viewed by a distant observer, predicted on the basis of the gravitational potential field. We calculate this field in the vicinity of the Local Group of galaxies from the observed peculiar (non-Hubble) velocities of galaxies, under the assumption that the peculiar motions are induced by gravity^{2–4}. If the cosmological density parameter Ω is 1, the gravitational potential field of the Great Attractor and surrounding regions produces a maximum Sachs–Wolfe anisotropy of $\Delta T/T = (1.7 \pm 0.3) \times 10^{-5}$ on an angular scale of 1° . Doppler and adiabatic contributions to this anisotropy are expected to be somewhat larger. If similar fluctuations in the gravitational potential are present elsewhere in the Universe, the anisotropy present when the CMB was last scattered should be visible from the Earth, and should be detectable in current experiments⁵. A fundamental test of whether gravity is responsible for the generation of structure in the Universe can be made by looking for the imprint in the CMB of deep potential wells similar to those found in our neighbourhood⁶.

Measurements of galaxy redshifts z and independent distances r yield radial peculiar velocities $u = cz - H_0 r$. (We assume hereafter that distances are measured in units of km s^{-1} so that $H_0 = 1$; c is the speed of light.) If the peculiar velocities are generated by gravity, and if the velocity field is smoothed sufficiently to remove small-scale nonlinear effects, the three-dimensional peculiar velocity is derived from a scalar potential: $\mathbf{v}(\mathbf{r}) = -\nabla\Phi(\mathbf{r})$. The velocity potential Φ may be recovered by integrating the smoothed radial velocity component along radial rays⁷

$$\Phi(\mathbf{r}) = - \int_0^r u(r', \theta, \phi) dr' \quad (1)$$

In the linear approximation, applicable on large scales, the peculiar gravitational potential $\phi(\mathbf{r})$ is proportional to the velocity potential³

$$\phi(\mathbf{r}) \approx \frac{3}{2}\Omega^{0.4}\Phi(\mathbf{r}) \quad (2)$$

Because the radial peculiar velocity data are smoothed on a scale of $1,200 \text{ km s}^{-1}$, the linear theory should be an excellent approximation³. The maximum smoothed density contrast found in the data⁴ is $\Omega^{0.6}\delta\rho/\rho = 1.2 \pm 0.4$ and the potential is less affected by nonlinearity than the density because it is weighted to larger scales. Tests have shown³ that current samples of spiral galaxies with infrared Tully–Fisher distance measurements^{7–10} and elliptical and S0 galaxies with D_n – σ (diameter–velocity dispersion) measurements^{11–13} are sufficient to recover the potential out to a distance $\leq 6,000 \text{ km s}^{-1}$ with a signal-to-noise ratio of nearly 10.

The gravitational potential field reconstructed from peculiar motions⁴ is dominated by a huge potential well, the ‘Great Attractor’^{11,14,15}. The flow towards the Great Attractor is

coherent over a scale of nearly $10,000 \text{ km s}^{-1}$, with a magnitude of $\sim 500 \text{ km s}^{-1}$. The corresponding maximum variation in gravitational potential is $\sim 8 \times 10^{-5} c^2$.

Gravitational potential fluctuations on the last-scattering surface cause CMB anisotropy through the Sachs–Wolfe and Doppler effects¹. For a matter-dominated universe, the CMB brightness-temperature fluctuation in direction $\mathbf{n}$ is

$$\frac{\Delta T}{T} = \frac{1}{3c^2} [\phi(\mathbf{x}_e) - \phi(\mathbf{x}_o)] - \frac{1}{c} \mathbf{n} \cdot (\mathbf{v}_e - \mathbf{v}_o) \quad (3)$$

where $\mathbf{x}_o$ and $\mathbf{v}_o$ are the observer’s comoving position and peculiar velocity and $\mathbf{x}_e$ and $\mathbf{v}_e$ are the comoving position on the last-scattering surface in the direction $\mathbf{n}$ and the effective peculiar velocity of the photon–baryon fluid there at decoupling. The first term, the gravitational redshift in an Einstein–de Sitter universe, is dominant on large scales whereas the second, the Doppler contribution, is important on small scales. For an $\Omega = 1$ dark matter-dominated universe the angular scale separating the two regimes is $\sim 2^\circ$. A comoving separation $|\Delta\mathbf{x}_e|$ on the last-scattering surface corresponds to an angular separation

$$\Delta\theta = 2 \arcsin(|\Delta\mathbf{x}_e|\Omega/4c) \quad (4)$$

or 1° for $|\Delta\mathbf{x}_e|\Omega = 10,500 \text{ km s}^{-1}$. The Sachs–Wolfe (gravitational redshift) anisotropy is general whereas the Doppler anisotropy, as well as the adiabatic contribution that we have neglected, depends on the amounts of baryonic and nonbaryonic matter and on the mode of perturbation (such as adiabatic or isocurvature)^{16,17}.

For $\Omega = 1$, in the linear regime, the gravitational potential remains constant in time. Consequently, the local gravitational potential derived from the present large-scale peculiar velocities has been unchanged since the redshift of the decoupling of radiation, $z_{\text{dec}} \approx 1,300$. We can therefore calculate the Sachs–Wolfe contribution to the CMB anisotropy using equations (1) and (2). The Doppler contribution may be estimated crudely by assuming that the photon–baryon fluid at decoupling had the same peculiar velocity field as the dark matter. These velocities are smaller than their present values by a factor $(1+z_{\text{dec}})^{-1/2}$. Radiation that was last scattered locally (long ago, but near our present comoving position) would now be reaching a distant observer nearly at the edge of our horizon. From equations (1)–(3) and the smoothed radial velocity field of nearby galaxies, we can predict the CMB anisotropy seen by such an observer.

For $\Omega \neq 1$ the calculations are complicated by the fact that the gravitational potential evolves with time as $\phi \propto D_1(t)/a(t)$,

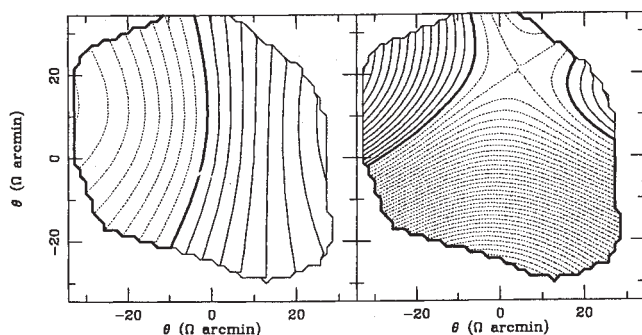


FIG. 1 Contour maps of $\Delta T/T$ for a distant observer who sees the last scattering of radiation occurring in the super-galactic plane. The left panel shows the Sachs–Wolfe contribution (present regardless of the dark matter and baryon abundance) and the right panel shows the Doppler contribution assuming that the photon–baryon fluid at decoupling has the same velocity as the dark matter (an overestimate). The contour spacing is $\Delta T/T = 10^{-6}$ with negative contours dotted and the zero contour (arbitrarily chosen) heavy. Regions with Sachs–Wolfe standard errors (arising from errors in the galaxy peculiar velocities) $> 2 \times 10^{-6}$, determined from Monte Carlo simulations, have been excluded.

where a is the cosmological expansion factor and D_L is the growth factor for the linear growing mode. Equation (3) still applies for the Sachs-Wolfe and Doppler anisotropies, but it neglects a contribution arising from the time evolution of the potential fluctuations at redshifts $z \ll |\Omega^{-1} - 1| - 1$. For $\Omega = 0.2$, the inferred Sachs-Wolfe anisotropy would be increased over our estimate by a factor of 1.56. The Doppler contribution is multiplied by an extra factor of $\Omega^{-1/2}$ so that, for $\Omega = 0.2$, it would be increased by a factor of 3.5. From equation (4), these increased anisotropies would be seen at a smaller angular scale because of the larger horizon distance ($\propto 1/\Omega$) in an open universe.

Figure 1 shows maps of the fluctuations in relative brightness temperature caused by the Sachs-Wolfe and Doppler effects for an observer looking at the local universe from the direction of the super-galactic north pole at nearly the horizon distance, assuming $\Omega = 1$. This observer sees our neighbourhood, including the Great Attractor (the potential well just beyond the left edge of the map), as it was at the time that the CMB radiation was last scattered. The finite thickness of the last-scattering surface has been accounted for approximately by first smoothing the potential with a three-dimensional gaussian of radius $1,200 \text{ km s}^{-1}$ or 7 arcmin. The intrinsic anisotropy has been smoothed by convolving each map with a two-dimensional gaussian beam of full width at half maximum (FWHM) 30 arcmin (for $\Omega = 1$) to mimic the experiment of Lubin *et al.*⁵, but no subtraction has been performed. If $\Omega < 1$, the angular scale would decrease and the amplitude of $\Delta T/T$ would increase as described above, with the model-dependent Doppler and adiabatic contributions dominating the Sachs-Wolfe anisotropy.

Although Fig. 1 shows the CMB anisotropy viewed by a distant observer in one small region of his last-scattering surface, the copernican principle argues that we should see similar regions of anisotropy on our last-scattering surface. We find the maximum Sachs-Wolfe anisotropy, caused by the Great Attractor, to be $\sim 1.7 \times 10^{-5}$ on a scale of 1° (for $\Omega = 1$ and a 30-arcmin beam; for a smaller beam the maximum anisotropy is slightly larger.) The maximum Doppler contribution, 4.9×10^{-5} , is overestimated because the photon-baryon fluid typically oscillates with a smaller velocity than the dark matter. For a cold dark matter-dominated universe with adiabatic perturbations and small baryon density the Doppler and adiabatic contributions on a scale of 1° typically double the Sachs-Wolfe anisotropy¹⁷.

Anisotropy as large as that shown in Fig. 1 would be seen only by a carefully placed observer with properly oriented beams directed right across the Great Attractor. The typical Sachs-Wolfe anisotropy on a 1° scale, based on the variation of the local gravitational potential field⁴, is $\sim 5 \times 10^{-6}$; the r.m.s.-smoothed $\Delta T/T$ at zero lag is 4×10^{-6} . The potential is determined accurately over a volume too small for us to determine the correlation function or the percentiles of the brightness-temperature fluctuations. Until accurate measurements of peculiar velocity are made for galaxies beyond $6,000 \text{ km s}^{-1}$, we cannot estimate how frequently anisotropies as large as 2×10^{-5} should be seen, but, unless we lie in a special place, anisotropies exceeding 10^{-5} should be fairly common.

If we live in an Einstein-de Sitter universe ($\Omega = 1$), the angular scale of the Great Attractor potential well, $\sim 1^\circ$ on the last-scattering surface, is an excellent match to the two-beam subtraction experiment of Lubin *et al.*⁵, with beams of 30 arcmin (FWHM) and a beam throw of 1° . Present anisotropy limits on this scale ($\Delta T/T \leq 3.5 \times 10^{-5}$, at 90% confidence¹⁷) are a factor of two or three above the Sachs-Wolfe anisotropies predicted here. These predictions require only the assumptions of general relativity applied to an approximately homogeneous and isotropic matter-dominated universe⁶. If gravitational instability is responsible for the generation of large-scale peculiar motions, and if our neighbourhood is not unique, the progenitors of large-scale structure should soon be detected in CMB anisotropy experiments. $\square$

Received 8 February; accepted 18 April 1990.

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ACKNOWLEDGEMENTS. We thank N. Vittorio and P. Lubin for discussions. This research was supported by NASA, the NSF, the US-Israeli Binational Science Foundation, and by an Alfred P. Sloan Foundation Fellowship to E.B.

The deep atmosphere of Venus revealed by high-resolution nightside spectra

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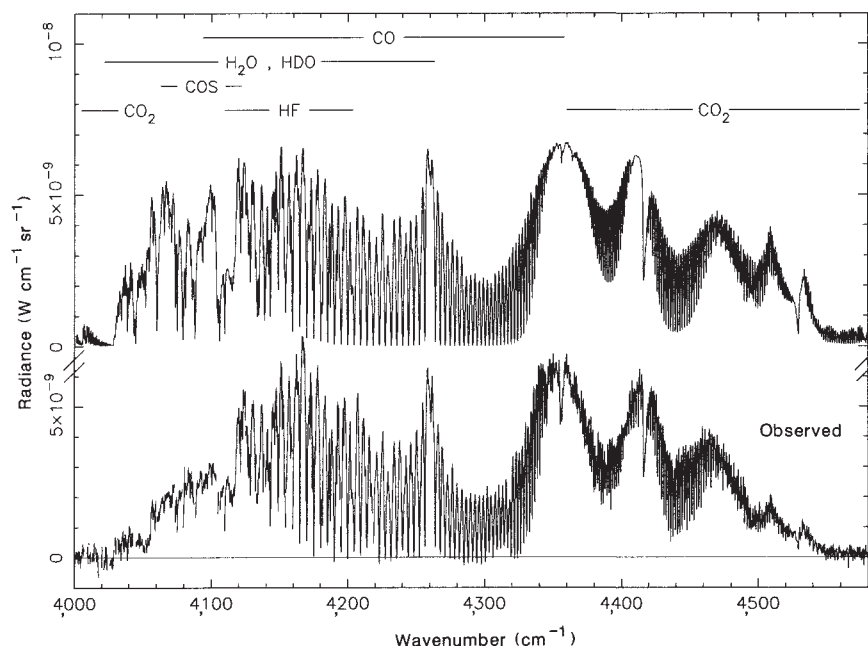
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THE night side of Venus is anomalously bright in two narrow spectral windows centred at 1.74 and 2.3 μm (ref. 1). The source of this emission has been identified recently as thermal radiation from atmospheric levels below the planet-wide decks of sulphuric acid cloud²⁻⁴. This emission provides a unique opportunity for remote-sensing investigations of the deep atmosphere of Venus. Here we report the first high-resolution (0.23 cm^{-1}) spectroscopic observations of the night side of Venus obtained in these two windows. Absorption features from CO_2 , CO , H_2O , HDO , HCl , HF and COS are detected, and there are a number of unidentified features. A preliminary analysis indicates that the observed radiation is thermal emission from atmospheric layers in the 8-bar pressure region for the 2.3- μm window and even deeper at 1.7 μm . The derived CO and H_2O abundances agree with *in situ* measurements by Pioneer in the deep troposphere. Our analysis is also consistent with the high deuterium enrichment inferred from Pioneer data. We report the first measurements of HCl and HF below the clouds, along with the first firm detection of COS . These results will assist in the interpretation of the data from the Galileo encounter with Venus, and will provide new constraints on theoretical models of the atmospheric chemistry of Venus.

In 1983, Allen and Crawford¹ recorded the first near-infrared images of the night side of Venus which revealed unexpected brightness in two wavelength ranges near 2.3 and 1.7 μm . The amplitude of this near-infrared emission varied over the disk, producing contrast features that seemed to rotate with a 6.5-day period. These spectral regions correspond to relatively transparent windows between strong absorption bands of the carbon dioxide and water vapour in the Venus atmosphere^{3,4}. Crisp *et al.*⁴ proposed that the near-infrared emission was produced as thermal emission from the deep atmosphere leaked through partial clearings in the planet-wide sulphuric acid clouds. Horizontal variations in cloud opacity are thought to be responsible for spatial variations in brightness. Kamp *et al.*³ analysed the low-resolution ($\lambda/\Delta\lambda = 100$) nightside spectra of Venus

FIG. 1 Bottom, 2.3- μm spectrum of the night side of Venus at an apodized resolution of 0.28 cm^{-1} . Data have been divided by a spectrum of the bright side recorded at a similar zenith angle to correct for telluric (Earth) opacity and filter response. Data corresponding to regions of strong telluric opacity are not plotted. top, Synthetic spectrum generated with the atmospheric model given in Table 1. Scattering and absorption by H_2SO_4 droplets is modelled through a wavelength-dependent cloud model that has an integrated extinction optical depth of 14 at $4,350\text{ cm}^{-1}$. Calculations include an additional continuum opacity parameterized with a constant absorption coefficient, $\alpha = 7 \times 10^{-8}\text{ cm}^{-1}\text{ amagat}^{-2}$ (see text).



recorded by Allen² and suggested that the abundances of CO and H_2O must be significantly less than those measured by Pioneer but their calculated spectra failed to reproduce several features in Allen's spectra.

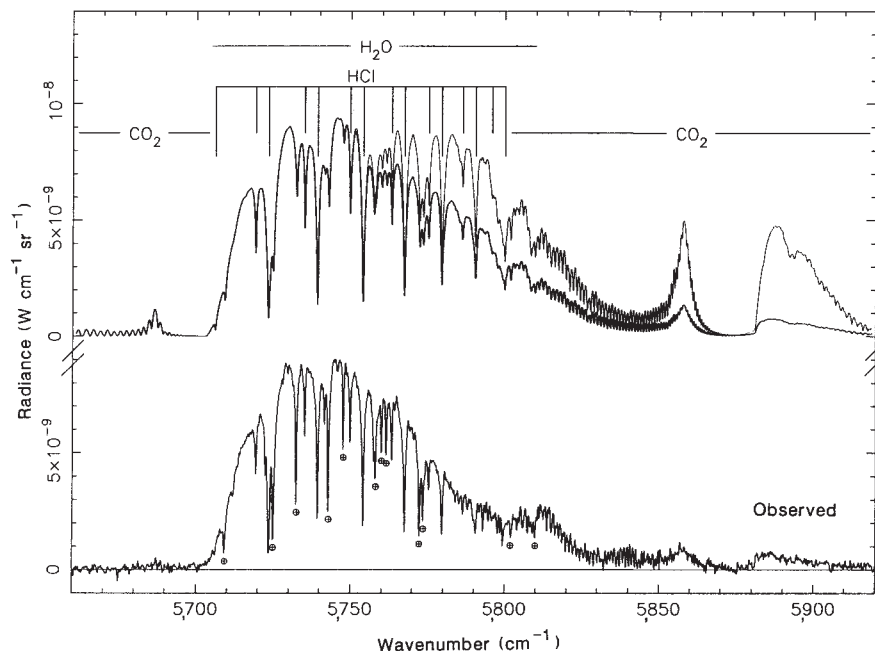
On 10 November 1989 (UT), we used the Canada-France-Hawaii telescope with the Cassegrain Fourier Transform Spectrometer to record high-resolution near-infrared spectra of the night side of Venus. A standard H and a wide K-blocking filter were used to isolate the 1.7- and 2.3- μm ranges, respectively. We centred a 5-arcsec circular aperture on a bright region located at $\sim 30^\circ\text{ N}$ on the dark side. In both spectral ranges, a resolution of 0.23 cm^{-1} (unapodized) was used. Three scans of nine minutes each were coadded to provide a single spectrum in the 2.3- μm region with a signal-to-noise ratio of 50. Only one 17-min scan was obtained in the 1.7- μm region, yielding a signal-to-noise of 100. Spectra of standard stars were recorded for absolute flux calibration. We removed some contamination by reflected sunlight from the day side of the planet which we found to amount to $\sim 10\%$ of the maximum signal observed in the two windows.

The maximum flux from the night side in the 2.3- μm range corresponds to blackbody radiation at 380 K, whereas the peak brightness temperature is 485 K at 1.74 μm . The Venus spectra are shown in Figs 1 and 2.

The low-frequency side of the 2.3- μm window is dominated by the $v=2 \rightarrow v=0$ band of CO (v , vibrational quantum number). A weak CO_2 band centred at $2.26\text{ }\mu\text{m}$ ($4,416\text{ cm}^{-1}$) is clearly visible on the high-frequency side of the window. Many H_2O and HDO absorption lines are seen below $4,300\text{ cm}^{-1}$. Four lines of the $v=1 \rightarrow v=0$ band of HF are also detected. The strong dip in the spectrum near $4,115\text{ cm}^{-1}$ is attributed to absorption from the $2\nu_3$ band of COS. In the 1.7- μm window, we detect 10 lines of the H^{35}Cl and H^{37}Cl isotopes. There is also some evidence for weak H_2O absorption lines on the high-frequency wings of their strong Doppler-shifted telluric counterparts.

To analyse these data, we calculated synthetic spectra using a line-by-line radiative-transfer program. A Delta-Eddington adding algorithm⁵ was used to solve the equation of transfer

FIG. 2 Bottom, 1.7- μm spectrum of the night side of Venus at an apodized resolution of 0.28 cm^{-1} . The data are not corrected for telluric absorption. Many telluric H_2O lines are thus present in the Venus spectrum, and are indicated as $\oplus$. top, Synthetic spectra generated with the atmospheric model of Table 1. First calculations (thin line) incorporate a constant value for the continuum absorption coefficient α ($7.7 \times 10^{-9}\text{ cm}^{-1}\text{ amagat}^{-2}$). A better fit to the shape of the observed spectrum is obtained by arbitrarily increasing α as $\alpha(\sigma)/\alpha(5,750\text{ cm}^{-1}) = e^{(\sigma-5,750)/90}$ for wavenumbers greater than $5,750\text{ cm}^{-1}$ (thick line).



with both gaseous absorption and particle scattering. The temperature profile is based on Venera and Pioneer probe data⁶. We used simplified cloud models compiled from Pioneer orbiter and descent-probe measurements⁵. The integrated cloud optical depth was kept as a free parameter, adjusted to match the observed flux level around $4,350\text{ cm}^{-1}$, a region relatively free of gaseous absorption. Spectra were calculated with a frequency step of 0.005 cm^{-1} , and then convolved at an apodized resolution of 0.28 cm^{-1} (full width at half maximum).

Spectroscopic data for CO, H₂O, HDO, HF and HCl were taken from the GEISA line compilation⁷. Line parameters for COS were generated from the molecular constants calculated by Fayt *et al.*⁸ and the band intensity determined by Kagann⁹. For CO₂ it was necessary to include weaker lines as well as those in the existing line catalogues^{7,10}. We therefore recalculated spectral-line parameters for transitions with J values (rotational quantum numbers) up to 90, using spectroscopic parameters provided by Rothman¹¹. We also added the weak $2\nu_3^{13}\text{C}^{16}\text{O}^{18}\text{O}$ band centred at $4,509\text{ cm}^{-1}$. This band eliminates the peak in the outgoing flux at $2.23\text{ }\mu\text{m}$ predicted by Kamp *et al.*³, and produces much better agreement with the spectra published by Allen².

Absorption in the wings of CO₂ lines is critical in our modelling because the spectral regions under investigation are highly transparent windows surrounded by relatively strong CO₂ bands. For all bands we used a sublorentzian lineshape based on the measurements of Burch *et al.*¹² in the $2.7\text{-}\mu\text{m}$ region, with an extrapolation further than 400 cm^{-1} from line centre, and assumed that this line-shape modification was independent of temperature.

Synthetic spectra generated with the opacity sources mentioned above cannot adequately reproduce the observed spectrum beyond $4,350\text{ cm}^{-1}$, where CO₂ is expected to be the only gaseous absorber (Fig. 3). The contrast in radiance is much too high, which suggests the presence of another continuum opacity source. It could be due to CO₂ itself, either because extreme wings of CO₂ lines are not as sublorentzian as assumed, or

TABLE 1 Vertical distributions of gases in the best-fit model

Molecule	Altitude (km)	Mixing ratio*
CO ₂		0.965
H ₂ O†	≤55	4×10^{-5}
	≥65	8×10^{-7}
CO	≤22	3×10^{-5}
	42	4.5×10^{-5}
	64	7.5×10^{-5}
	75	3×10^{-6}
	≥100	1.5×10^{-3}
HF		4.5×10^{-9}
HCl		5×10^{-7}
COS	≤50	2.5×10^{-7}
	≥65	1×10^{-8}

* Mixing ratios are interpolated linearly with altitude between the indicated levels.

† A D/H ratio 100 times the terrestrial value, as inferred from Pioneer measurements¹³, is assumed.

because of the presence of collision-induced CO₂ bands. We have therefore introduced another absorption coefficient in the form $\alpha p_{\text{CO}_2}^2$, where p_{CO_2} is the CO₂ number density, and chose $\alpha = 7 \times 10^{-8}\text{ cm}^{-1}\text{ amagat}^{-2}$ which provides the best fit to this part of the spectrum (Fig. 3). Unit optical depth resulting from this absorption is reached around the 8-bar level (32 km).

A synthetic spectrum was then calculated assuming that the same value of α applies to the whole $2.3\text{-}\mu\text{m}$ window. We first incorporated mixing-ratio profiles for the gaseous absorbers given in the 'standard' model of Kamp *et al.*³ which is based on probe data¹³. The best agreement with the observed spectra was obtained by increasing the CO mixing-ratio profile by a factor of 1.5. Fitting simultaneously the observed H₂O and HDO lines requires a reduction in the H₂O abundance by at least 20% and a strong enhancement of the D/H ratio over the terrestrial value (>50 times). The HF mixing ratio determined for the cloud tops by Connes *et al.*¹⁴ provides a good fit to our deep

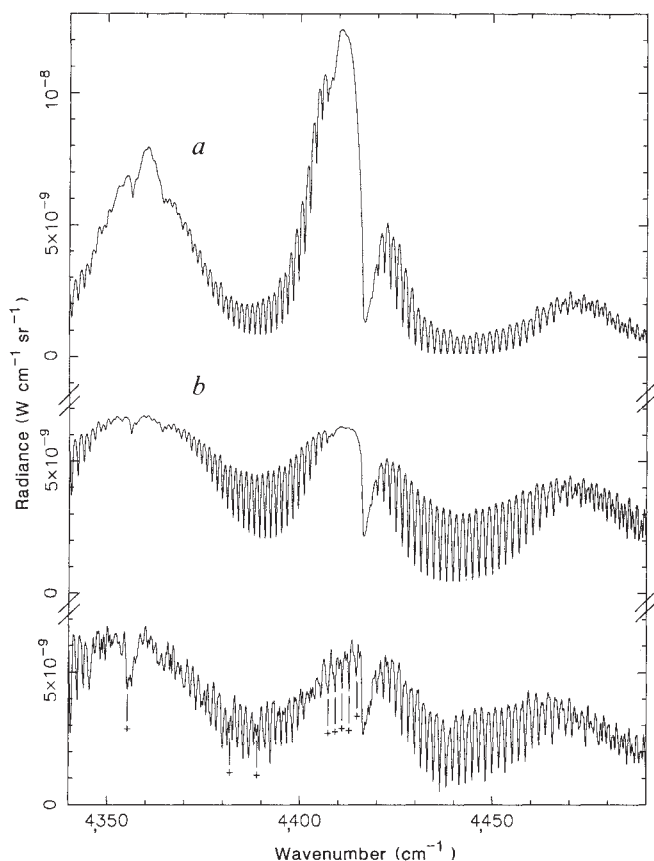


FIG. 3 Venus observations between $4,350$ and $4,500\text{ cm}^{-1}$, a region dominated by CO₂ absorption, are compared with synthetic spectra calculated with the atmospheric model of Table 1. In *a* no additional continuum opacity was included. In *b* a constant absorption coefficient ($\alpha = 7 \times 10^{-8}\text{ cm}^{-1}\text{ amagat}^{-2}$) was used. The latter yields a better fit to the observed spectrum, although a number of absorption features remain unidentified. The most intense ones are marked as (+). In each calculation, the total cloud optical depth is adjusted to match the observed radiance around $4,350\text{ cm}^{-1}$. Attenuation by the clouds, which occurs primarily above the line-forming region, has little influence on the line-to-continuum contrast.

atmospheric measurements. Our best-fit model is summarized in Table 1 and the corresponding spectrum is shown in Fig. 1. Our results differ from those of Kamp *et al.*³ who concluded that a reduction in the H₂O and CO abundances by a factor of 10 compared to their 'standard' model was needed. This difference is primarily a consequence of the use of a more complete description of the CO₂ spectrum in our model, and of an extra continuum opacity. These changes increase the gaseous opacity in our model and force the thermal emission to originate from higher altitudes. Larger amounts of H₂O and CO are therefore needed to produce the observed absorption along these shorter atmospheric paths.

The observed spectrum exhibits a drop in intensity around 4,115 cm⁻¹ which cannot be simulated using only the gaseous absorbers quoted above. This dip coincides with the R branch of the 2ν₃ band of COS, and can be correctly accounted for with a COS mole fraction of 2.5 × 10⁻⁷. This abundance is more than two orders of magnitude lower than that suggested by Venera 13 and 14 gas-chromatograph measurements¹⁵, but is compatible with the upper limit of 2 × 10⁻⁶ from the Pioneer measurements¹⁶. It is therefore likely that we report here the first true detection of this sulphur compound in the Venus atmosphere. There are still a few unidentified absorption features in the 2.3-μm spectrum. The sharp drop observed near 4,054 cm⁻¹ is near the 3ν₃-band centre of SO₂, but the lack of published spectroscopic data for this band has prevented us from investigating this possibility further. Some of the unassigned lines indicated in Fig. 3 may belong to weak CO₂ hot bands.

Spectra of the 1.7-μm region generated with a constant continuum absorption coefficient α fail to reproduce the shape of the observations (Fig. 2). There is evidence for a sharp increase in continuum absorption at wavenumbers $\geq 5,750$ cm⁻¹. We cannot therefore analyse the CO₂-dominated region to derive the altitudes from which radiation is emitted, as we did for the 2.3-μm region (see Fig. 3). To match the measured radiance at 5,745 cm⁻¹ with the cloud model derived from our 2.3-μm analysis, an absorption coefficient of 7.7 × 10⁻⁹ cm⁻¹ amagat⁻² would be required. The maximum pressure levels sounded in this spectral region would then be located around 25 bars (18 km). For this model, the observed depths of the HCl absorption lines can be correctly reproduced with a mixing ratio of $\sim 5 \times 10^{-7}$, a value similar to that measured in the cloud-top region by Connes *et al.*¹⁴.

This preliminary analysis shows that our measurements are representative of pressure levels below the clouds, down to 8 bars (32 km) for the 2.3-μm region, and deeper for the 1.7-μm region. We are therefore probing an altitude range in the Venus atmosphere that is particularly interesting because the abundances of trace chemical species there are influenced by photochemical processes in and above the clouds, thermochemical equilibrium, and interactions with the crust. Moreover, it includes the region below the clouds which is so critical for cloud chemistry. The results presented here could also have an important role in the interpretation of the data that were acquired by the Galileo spacecraft during its Venus flyby on 10 February 1990. The Near Infrared Mapping Spectrometer (NIMS) instrument on that spacecraft obtained near-infrared spectra for both the day and night sides of Venus. Their spatial resolution was ~ 30 –100 km, but their spectral resolution (0.025 μm) was much less than that described here. Our high-spectral-resolution results may improve the ability of the NIMS team to discriminate between the absorption by H₂O, and that of other gases. This must be done to produce maps of horizontal variations in the H₂O amounts in the atmosphere of Venus, which is an important goal of the Galileo encounter. □

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Meteoroid ablation processes in Titan's atmosphere

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SATURN'S planet-sized moon Titan has a nitrogen-rich atmosphere with a surface pressure of 1.6 bar (ref. 1). Methane, ethylene and other hydrocarbons are also present, allowing photochemical synthesis of complex organic molecules and aerosols^{2–6}. The ablation of meteoroids in Titan's upper atmosphere could contribute to the atmospheric chemistry in two important respects. First, refractory elements such as sodium, iron and magnesium could recondense into small (10–100 Å) particles which could act as aerosol nucleation centres, as in the Earth's atmosphere^{7,8}. Second, meteoroids from stray bodies with perihelia of ≥ 3 AU could be rich in volatile ices (H₂O, CO₂, CO), and might constitute an important source of oxygen-bearing material in Titan's atmosphere. Here I use a simple model of meteoroid ablation to calculate the altitude profiles of material introduced in this way from both icy and rocky meteoroids. The source region of oxygen-bearing molecules is found to lie between 600 and 800 km altitude; on Earth, by comparison, most of the meteoritic material evaporates between 80 and 100 km (refs 9, 10).

The presence of CO in Titan's stratosphere has been confirmed^{11–13}, and requires an exogenous source. Theoretical modelling of the chemical composition of Titan's atmosphere suggests that, on average, there should be a steady influx of the order of $\sim 6.1 \times 10^5$ water molecules cm⁻² s⁻¹ from meteoroid bombardment (ref. 4; M. Allen, personal communication). To understand the global chemistry and dynamical transport of the trace gases such as CO and possibly H₂O, the source distribution of the oxygen-bearing molecules must be clarified.

Here the equation of motion of the meteoroid under the influence of atmospheric drag is expressed as⁷

$$\frac{dv}{dt} = -\frac{3C_d\rho_a v^2}{4\rho_m R} \quad (1)$$

where v is the instantaneous velocity of the meteoroid at altitude Z , C_d the drag coefficient of order unity, ρ_a the atmospheric density, ρ_m the density of the meteoritic dust particle, and R is the meteoroid radius.

The kinetic energy loss through interaction with the atmosphere is balanced by the thermal radiation from the meteoroid surface, heating of the particle and surface sublimation or ablation

$$\xi \frac{\pi}{2} R^2 \rho_a v^3 = 4\pi R^2 \epsilon \sigma (T_m^4 - T^4) + \frac{4\pi}{3} R^3 \rho_m C \frac{dT_m}{dt} + L \dot{m} \quad (2)$$

where ξ is the energy transfer coefficient relating the efficiency of conversion of the meteoroid kinetic energy to heating, radiation and sublimation, ϵ is the emissivity of the meteoroid material, σ is Stefan's constant, C the heat capacity, T the

Received 13 March; accepted 11 April 1990.

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temperature of the atmosphere, and T_m the temperature of the meteoroid. I have assumed that $\xi \approx 0.2$ (ref. 14).

The temperature increase of the meteoroid as a function of flight time (and hence altitude) is determined from equation (2). The surface sublimation rate is then estimated by applying the Clausius-Clapeyron equation relating the vapour pressure P_v to T_m . The appropriate expressions for the Clausius-Clapeyron equations for ice and rocky material are given in ref. 15 from which I have also adopted the following physical parameters. For rocky material: $\rho_m = 3.4 \text{ g cm}^{-3}$, $C = 9.0 \times 10^6 \text{ erg K}^{-1} \text{ g}^{-1}$, $L = 8.1 \times 10^{10} \text{ erg g}^{-1}$, $T_b = 1,800 \text{ K}$. For icy material: $\rho_m = 1.0 \text{ g cm}^{-3}$, $C = 4.2 \times 10^7 \text{ erg K}^{-1} \text{ g}^{-1}$, $L = 2.8 \times 10^{10} \text{ erg g}^{-1}$, $T_b = 273 \text{ K}$. T_b denotes the temperature at which surface sublimation will become significant. In the numerical calculations, equations (1) and (2) are integrated with initial values of the meteoroid velocity V_0 and temperature T_0 at the starting altitude Z_0 . I also assumed that the meteoroid trajectory remains a straight line and that the entry angle (relative to the zenith) is $\chi = 45^\circ$.

The ablation profiles for a 100- μm meteoroid entering Titan's atmosphere are illustrated in Fig. 1. The mass vaporization rate as a function of altitude varies according to the initial relative velocity. For very high entry velocity ($V_0 = 40 \text{ km s}^{-1}$) the onset of surface sublimation of an icy particle occurs rather suddenly at $Z \approx 900 \text{ km}$. After reaching a peak value, the mass loss rate at lower altitudes slowly decreases because of the reduction in the size of the meteoroid. The icy meteoroid would be completely destroyed in flight before reaching altitudes below 650 km. For smaller incoming velocity, ablation begins to occur at lower

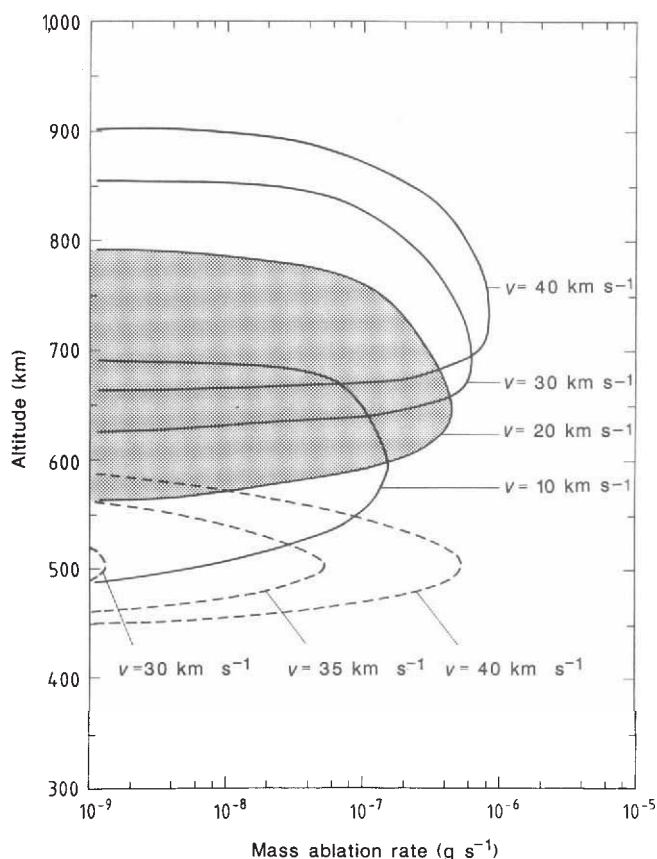


FIG. 1 The ablation zones for interplanetary meteoroids of 100- μm radius with different incoming velocity starting at an altitude of 2,000 km. The dashed curves are for rocky particles and the solid curves are for icy bodies. The entry angle from the zenith is assumed to be $\chi = 45^\circ$. The shaded region represents the meteoroid ablation zone expected for water-ice particles with atmospheric entry velocity near the high-velocity limit of 20 km s^{-1} at Titan.

altitudes. Because of the high volatility of the icy material, significant mass loss could still take place for V_0 as small as 5 km s^{-1} . The orbital distribution of the interplanetary meteoroids at Saturn's orbit is not known so it is difficult to estimate the actual distribution of the impact velocity. A plausible velocity range is $\langle V_0 \rangle = 15 \pm 5 \text{ km s}^{-1}$ when the presence of meteor streams from long-period comets in retrograde orbits is taken into consideration¹⁶. If this holds, icy particles of 100- μm radius are expected to deposit their mass at altitudes between 500 and 850 km.

For rocky meteoroids with $V_0 < 10 \text{ km s}^{-1}$, the continuous deceleration in the upper atmosphere is sufficient to slow down 100- μm particles such that the surface temperature is always smaller than T_b (1,800 K). No significant vaporization would occur for objects in such slow-moving trajectories. The main contribution would have come from particles with $V_0 \geq 20 \text{ km s}^{-1}$. I therefore expect the effective ablation zone of such rocky meteoroids to be found between 450 and 600 km altitude, just below the ablation region of the icy meteoroids.

If the size distributions of the icy and rocky meteoroids orbiting at 10 AU are similar to that found at 1 AU (see ref. 17), particles with $R_0 \approx 100 \mu\text{m}$ should be responsible for a large part of the mass influx to Titan's atmosphere. In this case, it is likely that at Titan the vertical distribution of metallic atoms and ions of meteoritic origin should be confined below 600 km altitude. The source region of the oxygen-bearing molecules would be located higher up, between 600 and 800 km altitude.

I have considered the possible consequences of meteoroid ablation in Titan's atmosphere for two different compositions of the interplanetary dust particles. The meteoroids might actually consist of a mixture of ice and rocky material such that the detailed mass vaporization profiles could be different from those described here. Also, the mass ratio of icy and rocky particles is unknown except for the requirement that the influx of oxygen-bearing material should be $\sim 6 \times 10^5 \text{ molecules cm}^{-2} \text{ s}^{-1}$ (ref. 4). A clarification of this important issue would require analyses of the compositions of interplanetary dust particles by dust instrument(s) in the environment of Saturn. Irrespective of the chemical nature of the interplanetary meteoroids, I expect the meteoritic ablation process to introduce two principal aeronomic effects in Titan's atmosphere in addition to the injection of oxygen-bearing material—the formation of smoke particles and the introduction of metallic atoms and ions in the upper atmosphere. Because the Na^+ and Fe^+ ions do not react strongly with N_2 and CH_4 (ref. 18), they might have a chance to accumulate, forming a metallic-ion-enriched layer. My calculations indicate that such a layer should have its maximum vertical altitude at about 500 km. Finally, it should be mentioned that the small metallic-ion clusters produced in the meteor vapour trails could have interesting chemical interactions with the organic hydrocarbon polymers. The ionosphere and photochemistry of Titan's atmosphere thus may be strongly coupled to the interplanetary medium. □

Received 12 February; accepted 17 April 1990.

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Regulation by light of methane emissions from a wetland

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WETLANDS provide up to 25% of the annual global flux of methane—an important greenhouse gas—to the atmosphere^{1–3}. Despite many studies^{4–9}, however, the factors that control emission from wetlands are uncertain. Methane production and transport by rooted vegetation^{10–15} have been emphasized, but methane oxidation has received little attention. Methane oxidation has been observed in a hardwood swamp⁷ and may consume >90% of the potential flux from rice paddies^{16–19}. Here I report results from a Danish wetland which suggest that methane oxidation is highly variable, consuming <10% to >90% of the potential methane flux. Laboratory experiments suggest that this variability may be due in part to light-mediated changes in oxygen distribution within illuminated sediments. These results indicate that diffusive fluxes of methane from wetlands may be controlled by rates of oxidation, in addition to rates of methane production. Failure to consider the dynamics and controls of methane oxidation in methane-flux studies could result in incorrect estimates of wetland emission rates.

The variability and photosensitivity of methane emission were evident in laboratory and field analyses including >10 separate experiments, some of which I summarize here. Intact cores obtained with or without a 1–2-mm-thick algal mat (primarily *Oscillatoria* spp.) exhibited higher rates of methane emission in the dark (Fig. 1). When an algal mat was present, dark emission

was 3.1–3.7 times that at a light intensity of $690 \mu\text{E m}^{-2} \text{s}^{-1}$ (E is the Einstein unit) for three sets of cores each run in triplicate. Methane emission from cores with no obvious algal mat was also photosensitive; in this case, rates were 1.5–3.1 times greater in the dark. In all cases, the response of methane emission to changes in light regimes was rapid (~ 20 min) and repeatable (Fig. 1). Steady-state emissions established under either a light or dark regime were also stable for periods up to 24 h. The rapidity and stability of the response suggested that the primary relationship involved a 'photostimulation' of methane oxidation and not changes in methane production.

To test the potential importance of changes in methane production, methane fluxes were measured for intact cores incubated in the dark either aerobically, anaerobically or aerobically with nitrapyrin (N-serv) added to the surface of the cores (details to be published elsewhere). Emission rates for the anaerobic and nitrapyrin-treated cores were the same and were 2.1 and 3.7 times greater than the aerobic controls in two trials. Because nitrapyrin inhibits both methane oxidation and production^{20,21}, and emission was equal in the nitrapyrin and anaerobic treatments, changes in methane oxidation and not methane production provide the best explanation for increased emissions in the treatments relative to the controls. This conclusion is also consistent with recent reports on the effects of varied oxygen on methane emission in a lake sediment²².

In a field experiment, methane emission was also significantly higher under dark conditions (Fig. 2). With ambient sunlight (~ 200 – $1,400 \mu\text{E m}^{-2} \text{s}^{-1}$), emission was $85.3 \pm 10.7 \text{ pmol cm}^{-2} \text{min}^{-1}$. During darkness, emission was ~ 1.8 times higher, $150.0 \pm 22.0 \text{ pmol cm}^{-2} \text{min}^{-1}$. These incubations were conducted when a thin film ($\ll 1$ mm) of various euglenophytes was present at the sediment surface and seemed comparable to

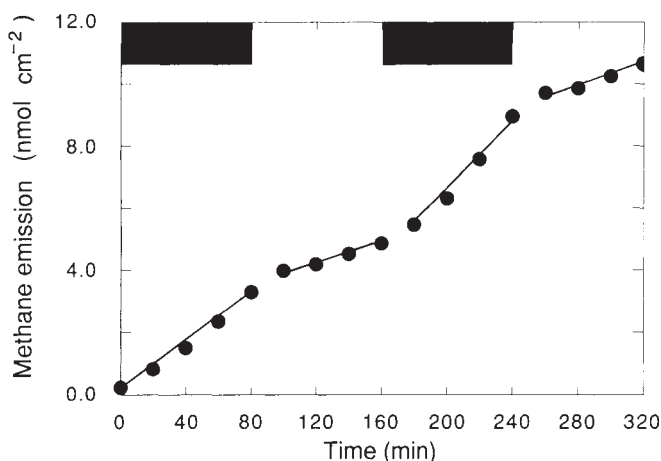


FIG. 1 Methane emission under alternating light and dark regimes in the laboratory. Triplicate acrylic core tubes (3.6-cm inner diameter) containing intact sediments with a 1–2-mm-thick algal mat at the sediment surface were sealed to provide a headspace of $\sim 60 \text{ cm}^3$. A fibre-optic light source with an infrared filter mounted through the top seal of the core tubes provided a light intensity of $690 \mu\text{E m}^{-2} \text{s}^{-1}$. The core tubes were wrapped with aluminium foil to prevent illumination by ambient light and to ensure darkness when the light source was off. Cores were incubated at $\sim 25^\circ\text{C}$. Samples ($100 \mu\text{l}$) of the core headspace were removed by syringe and needle at intervals for methane analysis by gas chromatography (using a Shimadzu GC-14A equipped with a flame-ionization detector and a capillary column (inner diameter 0.53 mm, 30 m long) containing DB-1). Rates of methane production under light and dark conditions were estimated by linear regression analysis. During the first dark period, methane emission (± 1 s.e.m.) was $38.3 \pm 2.1 \text{ pmol cm}^{-2} \text{min}^{-1}$ ($r^2=0.99$); during the second dark period the rate was $51.6 \pm 4.9 \text{ pmol cm}^{-2} \text{min}^{-1}$ ($r^2=0.97$). Rates during the two light periods, were 18.4 ± 2.1 and $18.4 \pm 2.6 \text{ pmol cm}^{-2} \text{min}^{-1}$ ($r^2=0.96$ and 0.94) respectively. Coefficients of variation for individual data points were 8.9–17.0%.

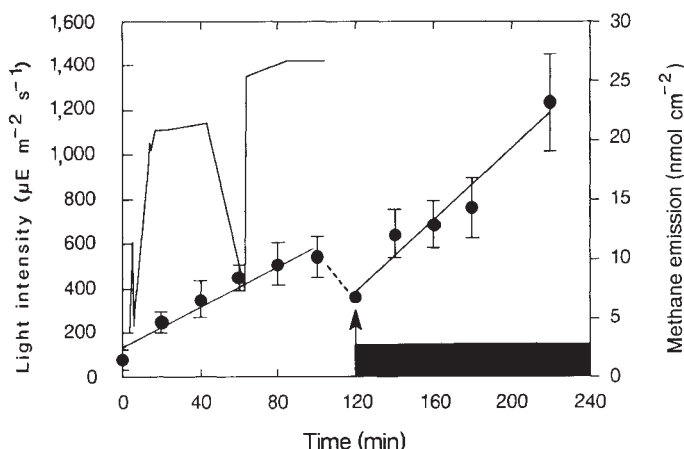


FIG. 2 Methane emission under light and dark regimes *in situ*. Clear acrylic chambers (3.6-cm inner diameter) were inserted into sediments overlain by ~ 3 cm of water and adjusted to provide a headspace of $\sim 100 \text{ cm}^3$. At the time of the analysis, no algal mats were obvious at the sediment surface. The chambers contained a small sampling port on the side that was sealed with a stopper from a 3-ml blood collection tube. Samples were removed by syringe and needle at 20-min intervals for analysis of methane by gas chromatography. The samples were stored without loss for a maximum of 4 h before analysis. The sediment surface was illuminated by ambient sunlight, the intensity of which (solid line, no symbols) was determined at intervals using a Li-Cor quantum meter (Model LI-185A). Ambient temperature was $\sim 18^\circ\text{C}$. Before darkening, the ports on the chambers were removed to re-equilibrate the chambers with the atmosphere (indicated by the arrow and dashed line). The chambers were covered with aluminium foil to provide dark conditions with continued sampling as before. Rates during each treatment were analysed by linear regression; coefficients of variation for individual data points were 11.8–30.5%. Error bars represent 1 s.e.m. Slopes of the light and dark emission rates were significantly different as analysed by a *t* test ($P < 0.05$).

TABLE 1 Relative extent of methane oxidation

Condition	Light oxidation (%)	Dark oxidation (%)	Light emission*
Algal mat present	77.3–82.0 (2)	16.4–44.2 (3)	84.3 ± 39.4
Algal mat absent	63.9–97.2 (4)	56.5–96.1 (5)	49.5 ± 26.0

Rates of methane emission were determined using intact cores incubated under aerobic illuminated, aerobic dark, and dark anaerobic conditions as described in the text and Figs 1, 3. The relative rates of methane oxidation were calculated from the differences between the aerobic and anoxic emission rates. The values given are ranges of the percentage oxidation observed during independent determinations (numbers in parentheses; each determination consists of triplicate cores). The light emission rates are means from the pooled independent experiments.

* Emission rates in $\text{pmol cm}^{-2} \text{min}^{-1}$.

laboratory incubations of cores with no distinct mats. The data indicated that the magnitude of the light effects were probably related to the extent and composition of the benthic algal community. A laboratory incubation of cores collected from the same site about two weeks after the field analyses showed an identical trend with dark emission rates 1.8 times higher than light emission rates. These results confirmed the utility and validity of the laboratory analyses of light-dark shifts.

Changes in light intensity from 0–1,090 $\mu\text{E m}^{-2} \text{s}^{-1}$ also resulted in changes in methane emission (Fig. 3). Rates of emission reached a minimum at $\sim 390 \mu\text{E m}^{-2} \text{s}^{-1}$. Therefore, trends determined under ambient field conditions or in the laboratory at $690 \mu\text{E m}^{-2} \text{s}^{-1}$ were comparable despite differences in light intensities. If expressed as potential rates of methane oxidation, the response to changing light intensities was similar to the

response of benthic photosynthesis^{23–25}. Potential oxidation rates were estimated as the difference between emission under aerobic and short-term (1–2 h) anoxic conditions. The similarity between the response of potential oxidation and photosynthesis is probably not the result of methane oxidation by photosynthetic organisms because only one such organism, *Rhodospirillum rubrum*, has been reported to oxidize methane²⁶. Furthermore, *R. gelatinosa* anaerobically photoassimilates limited amounts of methane into biomass, which is not consistent with the observed patterns or rates of methane consumption.

The effects of light-dark shifts on methane emissions are best explained as a result of changes in the availability of dissolved oxygen. Light-induced changes in benthic photosynthetic oxygen production are well known for their impact on both dissolved oxygen concentrations and rates of respiration, sulphide oxidation and redox-sensitive phosphorus fluxes^{27–30}. Here, typical patterns of increased oxygen penetration depths and concentrations under illuminated conditions were observed. For example, at $\sim 1,500 \mu\text{E m}^{-2} \text{s}^{-1}$, the oxygen penetration depth measured *in situ* was ~ 0.9 mm and a sub-surface oxygen concentration maximum of $380 \mu\text{M}$ occurred at 0.2 mm; during darkness the penetration depth halved to ~ 0.5 mm within 30 min and there was no sub-surface maximum (P. Kristensen and H. Skovgaard, personal communication).

When a distinct algal mat was present at the sediment surface, photosynthetic oxygen production was apparently a critical determinant of methane emission rates. Relative to anoxic controls, the potential methane flux decreased by 77–82% when sediments were illuminated; when dark, fluxes decreased only 16–44% (Table 1). Owing to the presence of readily degradable organic matter, heterotrophic oxygen uptake by the mat community may have constrained the availability of oxygen for methane oxidizers. When mats were absent, significant light-dark shifts still occurred but high rates of methane oxidation (up to 96% of the potential emission) may have continued in the dark because of a lower heterotrophic oxygen demand in the absence of high rates of primary production.

The results from this study indicate the importance of edaphic factors (that is, those related to local conditions in the sediment) as controls for methane emissions from wetlands. Aerobic methane oxidation is a dynamic process that is sensitive to changes in oxygen availability in freshwater ecosystems^{31,32}. Shifts in oxygen can occur rapidly because of changes in photosynthesis or respiration, leading to changes in methane emissions of several hundred per cent. Failure to consider the sensitivity of methane oxidation to parameters such as light regimes can result in inaccurate estimates of wetland methane emissions. As soils or sediments of most shallow freshwater systems support photosynthesis, light regimes may have an important role in determining the magnitude of emissions from one of the principal sources of atmospheric methane. Results from a recent analysis of peats of the Everglades National Park (Florida, USA) confirm the patterns described here (G.M.K. *et al.*, submitted, *Appl. env. Microbiol.*). □

Received 14 September 1989; accepted 9 April 1990.

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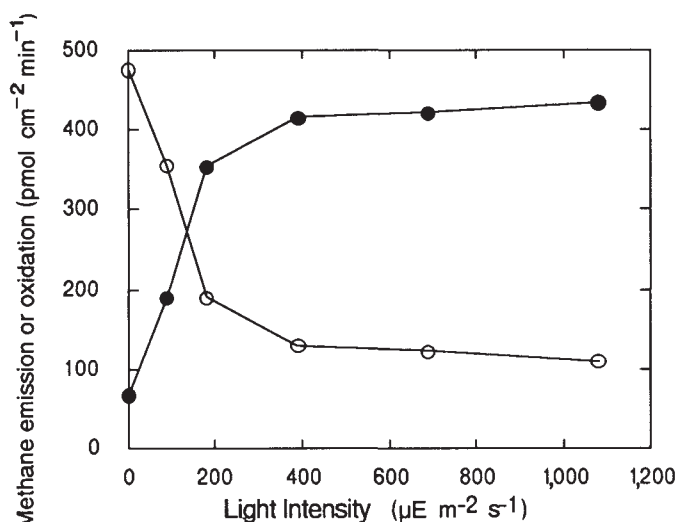


FIG. 3 Methane emission as a function of light intensity. Triplicate core tubes as described in Fig. 1 were subjected to stepwise changes in light intensity from a fibre-optic light source. Incubations were begun in the dark and intensities increased from 90 to $1,090 \mu\text{E m}^{-2} \text{s}^{-1}$; the incubation temperature was $\sim 25^\circ\text{C}$. Methane concentrations were analysed for ~ 80 min at each intensity to establish emission rates which were determined by linear regression analysis; all slopes were characterized by $r^2 > 0.98$. At the end of the light incubations, the cores were returned to darkness and emission measured for further 100 min. Rates of emission were not statistically different for the two dark incubations which were separated by ~ 8 h of light incubations. Immediately after the second dark incubation, the core headspaces were flushed with nitrogen. Methane emission was then measured under dark anoxic conditions for 120 min to establish a maximum potential rate of methane flux ($544.3 \pm 10.6 \text{ pmol cm}^{-2} \text{min}^{-1}$). Rates of methane oxidation (●) were calculated from the difference between the aerobic (light or dark) and the anoxic emissions. Coefficients of variation for individual data points were 2.3–15.7%.

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Non-activated magnetic relaxation in a high- T_c superconductor

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In the copper oxide superconductors the induced magnetic moments due to circulating supercurrents decay much more quickly than they do in most conventional superconductors¹. This inability to sustain strong persistent currents is an important limitation to technological applications of these materials. The quasi-logarithmic relaxation has been generally ascribed to thermally activated movements of magnetic flux lines². Here we report the results of relaxation measurements made at temperatures as low as 0.1 K in the critical state; we find that the decay process does not come to a halt as the temperature tends to zero. This means that a non-activated relaxation process dominates at low temperatures. Possible explanations include the tunnelling of flux lines through pinning barriers³, or the existence of an inhomogeneous distribution of critical current density in the sample⁴.

The sample was made up of monocrystalline grains of YBaCuO (sieved to a diameter of $\leq 30 \mu\text{m}$) in an epoxy matrix. Good c -axis alignment of the grains ($< 1^\circ$ spread) was obtained using a field of 8 T while the epoxy was curing at room temperature. We carried out two independent sets of relaxation experiments. For temperatures in the range 5–70 K magnetization relaxation measurements were made using a commercial high-field SQUID (superconducting quantum interference device) magnetometer in which the sample is moved between pick-up coils. We used the minimum possible travel length during the sample extraction to minimize spurious effects related to field inhomogeneity. For the low-temperature range (0.1–10 K) measurements were done with two Hall probes in a dilution refrigerator. In both sets of measurements the magnetic field applied along the c -axis of the grains and at each temperature the field was initially raised to 1 T and then reduced to 0.24 T, at which value the relaxation was monitored. Using this field cycle the sample was in the Bean critical state (maximum flux gradient throughout the sample) for the measurement, that is, the fields used were greater than both H^* (penetration field for the grains) and H_{c1} (lower critical field) at all temperatures. This is important because the form of the relaxation as a function of temperature is very different in small fields^{5–7}. The final field value was selected as being convenient for the Hall-probe measurements. The only drawback of this procedure was the inevitable creep of the field in the superconducting coil after field cycling (measured to be 2 G per time decade). This led to a small background term in the measurements, $\sim 10\%$ of the sample signal (see Fig. 1).

The temperature dependence of the effective logarithmic relaxation rate of the sample magnetization, $S(T) =$

$dM/d\ln(t)$, for t between 60 and 3,000 s is shown in Fig. 1. The SQUID data show a curve for $S(T)$ very similar to earlier results for YBaCuO samples in the critical state that covered the same temperature range^{5,6}, with $S(T)$ increasing progressively as the temperature is decreased. The data below 5 K show that $S(T)$ goes through a broad maximum near 3 K before dropping to a finite value as T tends to zero. The low-temperature variation seems to be approximately linear. Of particular interest is that $S(T)$ does not tend to zero at zero temperature as would be predicted by any thermal activation model.

Relaxation for high- T_c samples at a temperature well below 1 K has already been observed⁸, but the interpretation of the data was complicated both by the granular nature of the samples and the fact that the fields used were low so that the Bean critical state was not achieved. Relaxation near 1 K has also been observed in YBaCuO films⁹.

At temperatures down to 4 K the magnetization relaxation in copper oxide superconductors and in particular YBaCuO has been extensively studied. The data have usually been discussed in terms of the Anderson–Kim thermal activation model in which the relaxation is the result of movement of flux packets over pinning barriers under the driving force arising from a strong flux-line density gradient in the Bean critical state¹⁰. Because of the energy barrier all relaxation is expected to come to a halt at zero temperature. But the temperature dependence of the relaxation rate at temperatures down to 4 K cannot be fully understood using this approach^{4–6}, and our low-temperature results are further evidence of its inadequacy.

At least two distinct physical mechanisms invoking processes that are not thermally activated have been proposed, which could explain the observed behaviour. Mitin³ suggested the tunnelling of flux lines through pinning barriers to explain his data on relaxation below 1 K in a Chevrel-phase ($\text{Pb}_{1.2}\text{Mo}_6\text{S}_8$) compound. As in the YBaCuO, the coherence length in the

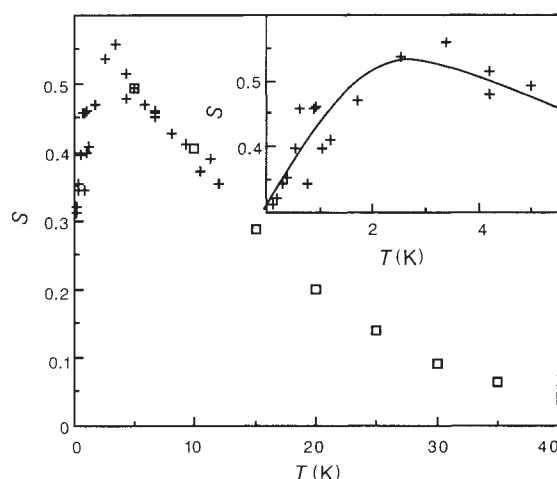


FIG. 1 The logarithmic decay rate of the magnetization, $S = dM/d\ln(t)$, as a function of temperature for the grain-aligned YBaCuO sample in the critical state with field along the c -axis. High-temperature points ($\square$), SQUID magnetometer; the low-temperature points ($+$), Hall probe. The scale of $S(T)$ is arbitrary and the two sets of data have been combined by scaling using the overlapping temperature region. The insert is an enlargement of the low-temperature region with a line as a guide to the eye. Low-temperature method: fixed geometry with two Hall probes (Siemens RHY18), one just below the sample and the other 5 cm away, and a modified a.c.-detection set-up¹², $f = 330 \text{ Hz}$, $I = 100 \mu\text{A}$. After correction for the different probe characteristics ($1.14 \Omega \text{ kG}^{-1}$ and $1.06 \Omega \text{ kG}^{-1}$) the difference signal is proportional to M . Measurements were done in a dilution refrigerator that could be stabilized between 0.1 K and 1 K or, when operated as a standard ^4He cryostat (with exchange gas and heater), between 1 K and 12 K in the same geometry. In a control experiment with an empty sample holder a small creep term was observed because of the weak creep in the field coils and the imperfect compensation of the Hall probes.

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Chevrel sample is small and Mitin proposed that the flux lines moved by a process in which short segments tunnelled through pinning barriers. The relaxation at zero temperature would then be a pure tunnelling effect whereas at finite temperature both tunnelling and thermal activation would contribute.

Alternatively, Sun *et al.*⁴ have discussed the magnetization relaxation in terms of an inhomogeneous distribution of critical current density in the sample, and point out a possible analogy to conventional superconductors in filamentary geometry¹¹. If the circulating screening current traverses a small part of the sample where the current present exceeds the local critical current, there will be dissipation locally until the current drops

below the local critical current level everywhere. A suitably chosen distribution of local current densities can lead to a screening current decay as observed. We have observed that the relaxation rate is strongly temperature dependent below 4 K, which would imply that the very small resistive regions have a strongly temperature-dependent resistance even at low temperatures.

Both the tunnelling and material-inhomogeneity models need to be developed further. If flux lines do tunnel through barriers, this would be an example of tunnelling in a quasi-macroscopic object. □

Received 9 April; accepted 8 May 1990.

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ACKNOWLEDGEMENTS. We thank J. J. Préjean for suggesting the Hall-probe technique and R. Cabanel for use of the SQUID magnetometer. A.H. acknowledges the support of the Department of Energy (US).

Hydrothermal scavenging of rare-earth elements in the ocean

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SUSPENDED particulate samples collected from the TAG hydrothermal vent field, at 26° N on the Mid-Atlantic Ridge, contain ~10% of the total rare-earth element content (by volume) of ambient sea water. Shale-normalized distribution patterns show both positive europium anomalies and negative cerium anomalies, indicating that the rare-earth elements in these hydrothermal precipitates come from both hydrothermal vent fluid and seawater sources. Rare-earth/Fe concentration ratios in the suspended particulate material increase at increasing distances from their hydrothermal source, indicating that rare-earth elements must be continuously extracted from sea water as hydrothermal precipitates are dispersed through the water column. Therefore, although high-temperature vent fluids escaping from the sea floor are typically enriched 10–10,000 times in rare-earth elements relative to sea water^{1–4}, hydrothermal systems must nevertheless act as a net sink in the global ocean budget of the rare-earth elements. But as the maximum rare-earth/Fe ratios observed for suspended particles

are ~10 times lower than previously reported values for hydrothermal sediments^{5–8}, it seems that most of the uptake of rare-earth elements from sea water must occur only after hydrothermal precipitates have settled to the underlying sediments.

We collected samples at distances ranging from <100 m to >1,000 m from the site of active venting using moored, transponder-navigated 'stand-alone' pumps. Samples were collected during RRS *Discovery* cruise 176 in July/August 1988 and analysed by inductively coupled plasma/mass spectrometry (ICP-MS) at MIT. Duplicate analyses of a representative selection of the samples, carried out by thermal ionization/mass spectrometry (TIMS) at the University of Cambridge, were in agreement within the precision of the ICP-MS technique ($\pm 10\%$, 2σ).

Distributions of particulate Fe concentrations in the neutrally buoyant hydrothermal plume at TAG are shown in Fig. 1. The highest particulate Fe concentrations occur at plume height (~300 m above sea floor) at the active vent site. Significantly lower particulate Fe concentrations occur in samples collected from 100 m below plume height. 'Background' samples with extremely low particulate Fe concentrations were obtained by deploying pumps at plume height in an area free from hydrothermal influence and also by deploying a pump some 400 m above the plume in the active vent field. With the exception of these three background samples the suspended particulate material consisted almost entirely of iron oxyhydroxides ($[\text{Fe}]/[\text{Fe} + \text{Al} + \text{Mn}] > 95\%$).

Particulate Fe and rare-earth element (REE) concentrations are listed in Table 1. Maximum particulate Fe concentrations in the neutrally buoyant plume were ~210 nmol l⁻¹ whereas

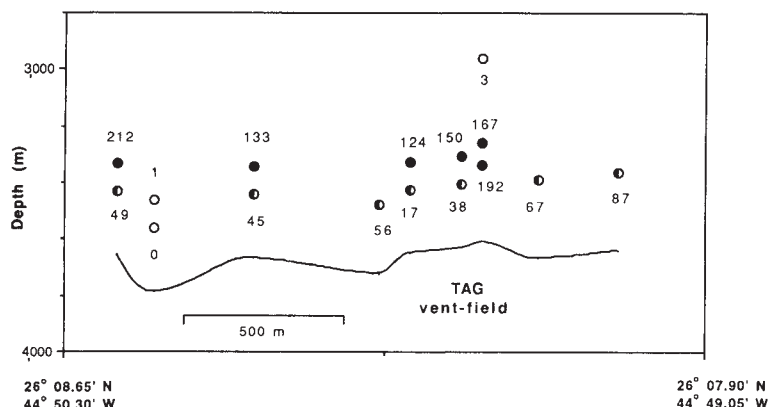


FIG. 1 Vertical section (northwest-southeast) indicating particulate Fe concentrations in and below the neutrally buoyant hydrothermal plume at TAG, 26° N, Mid-Atlantic Ridge. Solid circles: $[\text{Fe}]_s > 100 \text{ nmol l}^{-1}$ sea water. Half-filled circles: $10 \text{ nmol l}^{-1} < [\text{Fe}]_s < 100 \text{ nmol l}^{-1}$. Open circles: $[\text{Fe}]_s < 10 \text{ nmol l}^{-1}$. Numbers beside points indicate the exact concentrations (nmol l⁻¹).

TABLE 1 Particulate REEs and Fe

Sample	Fe (nmol l ⁻¹)	La	Ce	Pr	Nd	Sm	Eu	Gd	Tb	Ho	Er
		(pmol l ⁻¹)									
TAG:14	56	1.90	1.83	0.465	1.87	0.474	0.111	0.383	0.065	0.072	0.194
TAG:18	87	2.68	2.02	0.683	2.81	0.668	0.168	0.559	0.101	0.113	0.301
TAG:19	67	2.13	1.69	0.528	2.16	0.509	0.130	0.446	0.077	0.087	0.226
TAG:22	192	3.96	2.26	1.009	4.09	0.893	0.265	0.753	0.150	0.179	0.469
TAG:32T	212	4.14	2.48	1.039	4.20	1.001	0.265	0.813	0.149	0.165	0.434
TAG:32B	49	1.99	1.97	0.522	2.02	0.456	0.119	0.387	0.069	0.074	0.184
TAG:35T	133	3.73	2.14	0.942	3.78	0.872	0.237	0.775	0.138	0.155	0.413
TAG:35B	45	1.69	1.78	0.437	1.71	0.377	0.090	0.315	0.060	0.067	0.174
TAG:39T	150	3.70	2.06	0.944	3.85	0.847	0.229	0.756	0.143	0.165	0.441
TAG:39B	38	1.49	1.68	0.390	1.62	0.364	0.092	0.308	0.055	0.062	0.160
TAG:43T	124	3.24	2.15	0.823	3.35	0.754	0.213	0.613	0.121	0.138	0.365
TAG:43B	17	0.98	1.61	0.253	1.02	0.224	0.057	0.194	0.035	0.035	0.093
TAG:48T	1	0.70	1.59	0.157	0.62	0.127	0.032	0.112	0.017	0.018	0.044
TAG:48B	0	0.65	1.46	0.148	0.59	0.122	0.026	0.111	0.017	0.017	0.046
TAG:53T	3	0.72	1.45	0.160	0.63	0.144	0.032	0.133	0.019	0.020	0.056
TAG:53B	167	3.55	2.34	0.932	3.98	0.884	0.263	0.733	0.145	0.170	0.445
Sea water		29.35	7.26	4.87	20.66	4.13	1.047	5.12	0.795	1.554	4.97
Vent fluid	2 × 10 ⁶	2,700	5,800	750	2,700	470	2,600	390	69	34	70

maximum dissolved Mn concentrations measured in the same study were $\sim 60 \text{ nmol l}^{-1}$ (RRS *Discovery*, cruise 176, unpublished data). Because dissolved Fe concentrations were below the detection limit of $\sim 10 \text{ nmol l}^{-1}$ at plume height (RRS *Discovery*, cruise 176, unpublished data) and particulate Mn concentrations were $< 1 \text{ nmol l}^{-1}$ throughout (C.R.G., unpublished data), a total Fe/total Mn ratio at plume height of $\sim 3.5:1$ can be calculated. By contrast, endmember hydrothermal-vent fluids collected from TAG with DSRV *Alvin* in January 1990 had a total (dissolved) Fe/Mn ratio of $\sim 10:1$ (M. J. Greaves and A. C. Campbell, personal communication). From a comparison with ^3He distributions, it has been demonstrated that dissolved Mn behaves conservatively within 1–2 km of the site of eruption at the TAG hydrothermal vent field (M. Rudnicki, personal communication). Consequently, any fractionation of Fe from Mn between eruption and emplacement in the neutrally buoyant plume can only be effected by preferential removal of Fe in the buoyant portion of the plume, presumably through precipitation as sulphides deposited close to the vent orifice⁹. Because the Fe/Mn ratio in the neutrally buoyant plume is about three times lower than that for the endmember fluids, we conclude that about two-thirds of the dissolved Fe erupted at TAG must be removed in this way.

Particulate REE/Fe ratios in the neutrally buoyant plume represent an enrichment greater than tenfold, relative to endmember vent fluids (for example, $\text{Nd/Fe}_{\text{particles}} = 2\text{--}6 \times 10^{-2} \text{ pmol nmol}^{-1}$; $\text{Nd/Fe}_{\text{vent fluids}} < 2 \times 10^{-3}$; Table 1). In the extreme case, precipitation of Fe-rich sulphides without any accompanying removal of REEs from solution could account for a maximum REE/Fe enrichment of $\sim 3:1$ relative to the vent fluids. Therefore, although precipitation and deposition of Fe-rich, REE-poor sulphides may account for some of the enrichment apparent in the neutrally buoyant hydrothermal plume, most of the enrichment ($\geq 65\%$) must come from other sources.

Maximum particulate REE concentrations in the samples analysed represent $\sim 10\%$ of ambient dissolved REE concentrations (Table 1), a much higher proportion than is typical of the deep ocean¹⁰. Shale-normalized¹¹ REE distribution patterns for these particulate samples (Fig. 2a) show both negative Ce anomalies, characteristic of sea water, and positive Eu anomalies, typical of hydrothermal vent fluids. These features are observed for all samples collected in the hydrothermal plume indicating that the REEs in these samples derive from both sea water and hydrothermal sources. By contrast, the three background samples exhibit extremely flat shale-normalized REE

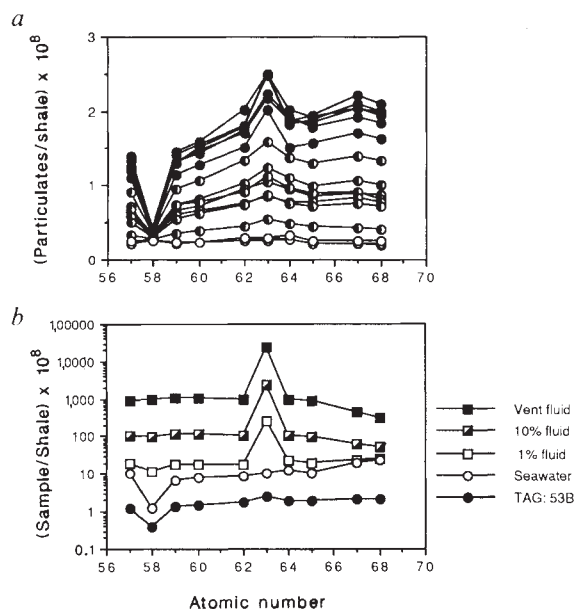


FIG. 2 a, Shale-normalized REE distribution patterns for suspended particulate samples from the neutrally buoyant plume overlying the TAG hydrothermal field (symbols as in Fig. 1). b, Shale-normalized REE distribution patterns for hydrothermal vent fluids, calculated hydrothermal fluid/seawater mixtures and ambient sea water from the Mid-Atlantic Ridge rift valley (logarithmic scale). The distribution pattern for particulate sample TAG:53B (uppermost sample in a) is shown for comparison.

distribution patterns suggesting that these samples represent small amounts of ambient detrital aluminosilicate material settling through the water column.

The distinctive nature of shale-normalized REE distribution patterns for sea water and hydrothermal vent fluids allows the relative contributions of these two sources to the hydrothermal particulates to be deduced. In Fig. 2b we present shale-normalized REE distribution patterns for endmember hydrothermal fluids and ambient sea water from the Mid-Atlantic Ridge. Because pure endmember vent-fluid analyses are not yet available from the TAG hydrothermal field, REE distributions for endmember fluids from the MARK site at 23° N, Mid-Atlantic Ridge⁴ have been used. Also presented are calculated distribu-

tion patterns for the mixtures (10% hydrothermal fluid, 90% sea water) and (1% hydrothermal fluid, 99% sea water). Both of these calculated distribution patterns yield pronounced positive Eu anomalies, but little or no negative Ce anomaly is apparent. This is quite unlike the trends observed for iron oxyhydroxide samples collected from directly above the vent field. In fact, distribution patterns similar to those of our samples can only be generated by a mixture with the proportions 0.1% hydrothermal fluid, 99.9% sea water. Total concentrations of particulate REEs comparable to those reported here could then be generated by removal of ~10% of the dissolved REEs from such a mixture. This would represent incorporation of ~0.1 p.p.t. vent-fluid REE concentrations into the particles, comparable to the ~0.1 p.p.t. particulate Fe concentrations reported earlier. But these particulate REE concentrations would also represent incorporation of some 10% of the dissolved REE content of ambient sea water. Evidently, although high-temperature vent fluids are typically 10–10,000-fold enriched in REEs relative to sea water^{1–4}, vigorous scavenging determines that hydrothermal systems must act as a net sink in the global ocean budget of the REEs. This confirms the hypothesis proposed from a previous study of dissolved REE concentrations in sea water close to hydrothermal vents in the southeast Pacific Ocean¹².

Further, although particulate REE concentrations and particulate Fe concentrations are strongly correlated throughout the hydrothermal plume (Fig. 3) the trends observed here are not those that would result from simple coprecipitation at source and subsequent dilution with ambient sea water. Such a process would be expected to result in strictly linear trends for variations with particulate Fe concentrations as have been reported previously for several other species (such as V, As, PO₄, Cr, U)^{13–16}. Instead, our plots of particulate REE against particulate Fe show a significant positive departure from such a simple linear mixing line. Therefore, in addition to initial coprecipitation and uptake by hydrothermal particles, further scavenging of the REEs must occur during dispersion of these particles through the water column. Consequently, the REE/Fe ratio of suspended hydrothermal particles is observed to increase at increasing distance from the active vent field.

The highest REE/Fe ratios observed in this study, however, (for example, Nd/Fe_{particles} = 0.06 pmol nmol⁻¹) remain some 5 to 50 times lower than those reported previously for the hydrothermal component of metalliferous sediments from the East Pacific Rise and southern Juan da Fuca Ridge^{5–8}. Our observations support the hypothesis from these previous studies that significant scavenging of the REE must occur only after deposition of suspended hydrothermal precipitates from the water column. Consequently, the hydrothermally derived component of the REE should become progressively obscured with increasing age and shale-normalized distribution patterns for metalliferous sediments should increasingly reflect the REE pattern of overlying sea water. □

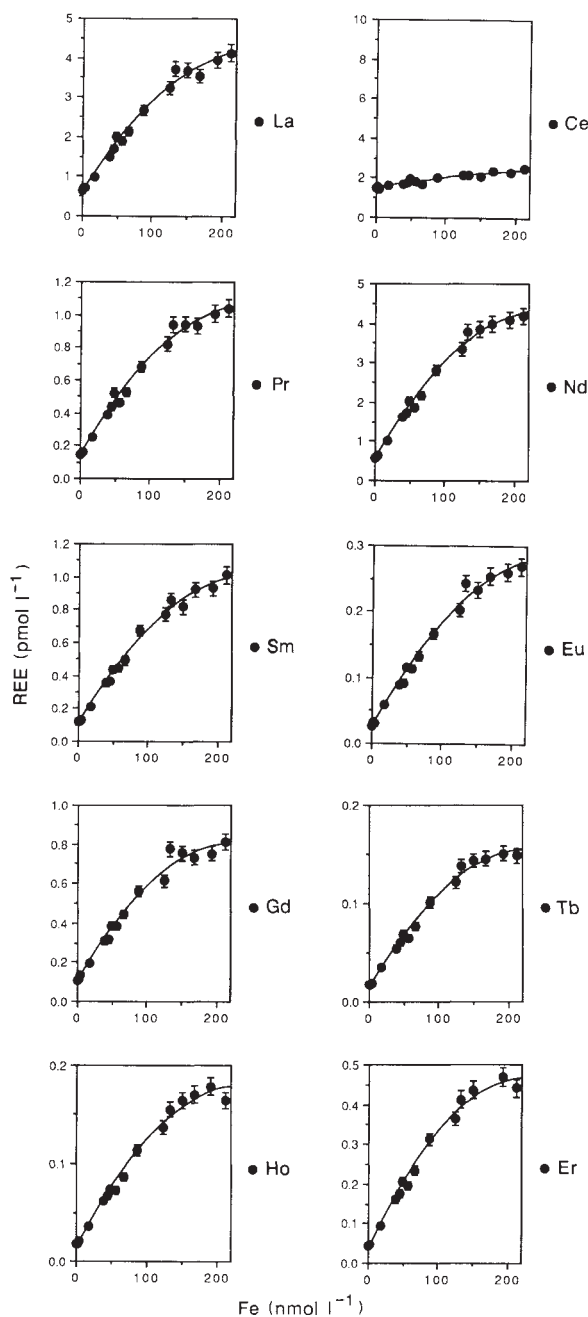


FIG. 3 Particulate REE concentrations plotted against particulate Fe concentrations for samples from the neutrally buoyant plume overlying the TAG hydrothermal field.

Received 2 January; accepted 12 April 1990.

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ACKNOWLEDGEMENTS. We thank W. R. Simpson, L. Wright and N. Hooker from IOS Deacon Laboratories, P. Taylor of RVS Barry and L. Godfrey for support in the operation of the stand-alone pumps. The ICP-MS laboratory at MIT has been established primarily by K. K. Falkner with grants from the NSF awarded to J. M. E. Our research has also been supported by the Royal Society, NATO and NERC.

Influence of magma withdrawal on compositional gradients during the AD 79 Vesuvius eruption

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COMPOSITIONAL variations in pyroclastic deposits have been used as indicators of pre-eruptive chemical gradients in magma chambers and of processes leading to compositional stratification^{1,2}. Theoretical studies of the fluid dynamics of magma withdrawal from stratified chambers have shown that compositional gradients in the products erupted at the surface are not merely inversions of the gradients that had existed in the magma chamber. These studies predict that complex mixing can occur across stratified layers, and that withdrawal of dense underlying layers through overlying low-density layers can occur given certain combinations of discharge rate and magma density contrast³⁻⁷. These predictions have yet to be quantitatively verified, however, because simultaneous measurements of eruption dynamics and compositional gradients for a specific eruption have not been collected. Here we explore the relationship between chemical gradients observed in pyroclastic deposits and the dynamics of magma withdrawal, by modelling the temporal evolution of magma discharge rate during the AD 79 eruption of Vesuvius in Italy. The results provide evidence that compositional gradients in zoned deposits cannot be properly interpreted without consideration of the dynamics of magma withdrawal.

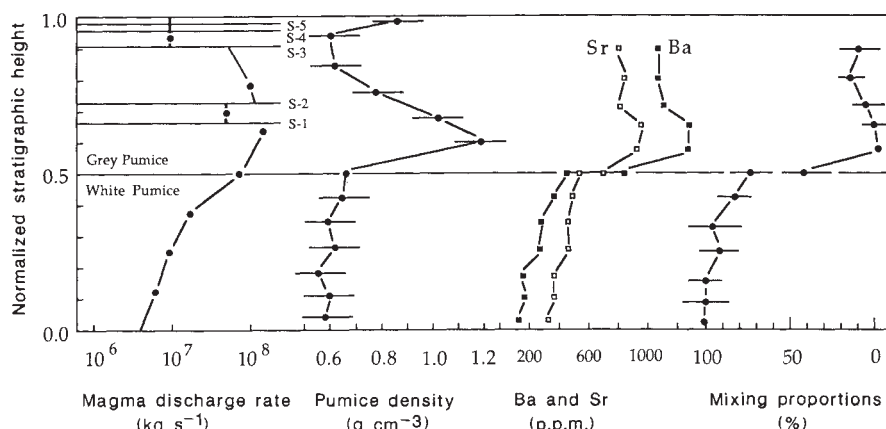
The eruption of Vesuvius in AD 79 is well known for its burial of the Roman cities of Pompeii and Herculaneum and is a well known example of a plinian explosive eruption⁸. About 4 km³ of phonolitic magma was ejected over a period of 19 hours on 24 August AD 79⁹. A change in magma composition during the eruption is marked by an abrupt transition from white evolved pumice to denser grey, less evolved pumice at mid-level in the deposit (Fig. 1). During later stages of the eruption, fallout of grey pumice was interrupted five times by pyroclastic flows and surges. Variations in magma discharge rate were calculated by measuring changes in dispersal characteristics of the fall deposit as a function of eruption duration or stratigraphic height^{10,11}. During the first part of the eruption, evolved phonolite magma

(white pumice), was ejected with a rate increasing from 5×10^6 to 8×10^7 kg s⁻¹ (Fig. 1). After seven hours of plinian activity, the magma composition changed abruptly to mafic phonolite (grey pumice) but the magma discharge rate continued to increase to a peak value of 1.4×10^8 kg s⁻¹. At this time the first pyroclastic flow and surge (S-1) were generated⁸.

The variation in eruption rate is paralleled to a remarkable degree by variations in pumice density and chemical composition (Fig. 1). Pumice density varies by a factor of two and largely reflects differences in vesicularity⁹. Chemical variations, as exemplified by Sr and Ba show a peak just above the white/grey boundary and a reduction in the final stages. These trends are consistent with the mixing of two endmembers, as shown by the linearity of a plot of Ba/Sr against 1/Sr (Fig. 2). The endmembers correspond to evolved phonolite magma (white pumice) and a later-erupted, more primitive mafic phonolite. The exact composition of the primitive endmember cannot be precisely defined, however, because all grey pumice has been affected by mixing to some extent. Microprobe analyses of glassy melt inclusions in phenocrysts indicate that there were two dominant magma types in the reservoir before eruption and no continuous compositional gradient is recorded by the inclusions. We have estimated the water content of the two magma types by the 'difference' method using melt-inclusion analyses and found values of 4.7 and 3.5 ± 0.50 wt% for the white and grey pumice, respectively¹².

Physical evidence for the mixing of magma is found throughout the grey pumice, which contains sanidine crystals wetted by 20 to 100- μ m coatings of evolved phonolitic magma similar to that which forms the white pumice¹². In addition, phenocrysts that are the equilibrium assemblage in the white evolved phonolite commonly occur in the grey pumice. We have found no such mineralogical evidence for mixing in the white pumices, however, despite the fact that they also show a linear trend in Fig. 2. The compositional trends observed in the white pumice are thus consistent with either two endmember mixing or a slight pre-eruption chemical gradient in the white magma. We therefore propose that some mixing of the endmembers occurred at the grey-white pumice transition, as is evident from the presence of rare banded pumices at this level (0.5 level, Fig. 1). Further mixing of the two endmembers occurred after the first appearance of the grey mafic phonolite at the normalized stratigraphic level of about 0.6 (Fig. 1). Mass balance calculations indicate that the proportion of evolved magma increased up to 20% in the mixed magma after the peak discharge rate (Fig. 1). Thus the compositional gradient in the grey pumice primarily reflects varying degrees of mixing between two magma

FIG. 1 Variation in magma discharge rate, pumice density, Ba and Sr content of pumice, and mixing proportions during the plinian stage of the AD 79 eruption of Vesuvius, shown as a function of normalized stratigraphic height in the pumice fallout deposit⁸. Magma discharge rate (kg s⁻¹) was determined from clast size distribution in the fallout deposit as a function of distance from the vent⁹. Horizontal lines labelled S-1 to S-5 correspond to the stratigraphic position of intraplinian surge deposits. Variation in pumice density reflects vesicularity and therefore volatile content of the magma tapped. Mixing proportions represent the amount of mafic phonolite magma that is required to be mixed with the evolved phonolite to produce the observed chemical gradient, based on mass balance calculations using trace and major-element chemistry of whole rocks and glasses¹². The apparent mixing trend in the white pumice may be fortuitous, and this part of the trend may reflect a pre-eruption compositional gradient. Error bars in mixing-proportion data are the range in proportions calculated from both trace and major elements. The error in Sr and Ba determinations is less than the size of the data points (2σ).



types and the extent of mixing seems to be closely linked to variations in magma discharge rate (Fig. 1). With exception of rare banded pumices, the magmas are very thoroughly mixed before fragmentation, as indicated by chemical homogeneity of the glasses at each stratigraphic level.

The geochemical evidence reflects withdrawal of magma from a compositionally stratified reservoir consisting of a layer of evolved low-density volatile-enriched magma overlying a layer of denser and more primitive, less volatile-rich magma (Fig. 3). When a given combination of magma discharge rate, magma density contrast and kinematic viscosity is attained, withdrawal of magma from a two-layer stratified reservoir can result in the drawing up of magma from the lower layer through the upper layer⁵. For the AD 79 eruption, with high magma discharge rate and relatively high kinematic viscosity, the drawing up of magma would have been largely in the viscous-flow regime, and determined by viscous and buoyancy forces^{5,6}.

In this situation, the maximum depth from which the primitive magma can be withdrawn through the overlying evolved layer is given by:

$$d = c(Q\nu/g')^{0.25} \quad (1)$$

where d is the draw-up depth (m), c is a constant (2.42) for flow conditions in the viscous regime^{5,6}, Q is volume eruption rate ($\text{m}^3 \text{s}^{-1}$) determined from magma discharge rate, ν is kinematic viscosity of the upper layer ($9.3 \text{ m}^2 \text{s}^{-1}$, determined from chemical composition, crystal content and temperature)¹² and $g' = g(\rho_2 - \rho_1)/\rho_1$, with ρ_1 and ρ_2 the densities of the upper and lower layers ($2,380$ and $2,510 \text{ kg m}^{-3}$, respectively)¹² and g is the acceleration due to gravity. Using equation (1) and the appropriate values for endmember compositions, we find that the value of d increased from 40 to 80 m with increasing magma discharge rate, and then decreased to 40 m at the end of the event. These calculations are based on assumption of steady flow conditions. Conditions during the eruption differ from theoretical modelling. The magma discharge rate varied by an order of magnitude, whereas current models have been developed for conditions of constant magma discharge rate. Also, because of higher crystal content, the lower layer in the Vesuvius magma reservoir has the higher viscosity¹². Experimental results show, however, that draw-up depth is independent of lower-layer viscosity⁵.

In the early stages of the eruption, the draw-up depth increased from 40 to 50 m, and only evolved magma was erupted. Although the thickness of the upper (evolved) magma layer was steadily decreasing, the draw-up depth was not great enough to intercept the boundary between evolved and primitive magmas.

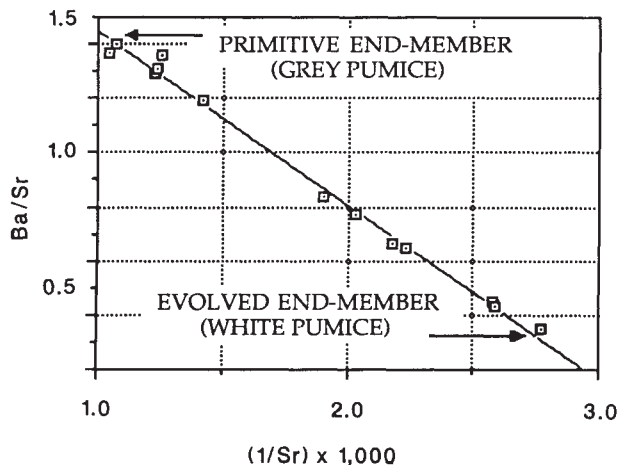


FIG. 2 Plot of Ba/Sr against $(1/\text{Sr}) \times 1,000$ for pumice of the AD 79 fall deposit at Pompeii. The compositional range is well accounted for by a two-component mixing model of the primitive and evolved endmembers, as shown by the mixing line.

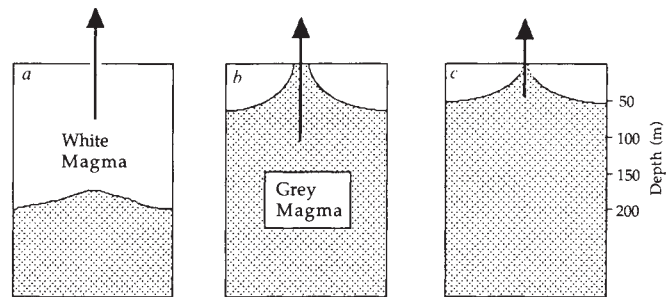


FIG. 3 Diagrammatic representation of the withdrawal of magma from the Vesuvius chamber during the AD 79 eruption. In early stages of the eruption (a) the withdrawal depth (40 m) was less than the thickness of the evolved magma layer and only white pumice was erupted (maximum discharge rate $= 5 \times 10^6 - 8 \times 10^7 \text{ kg s}^{-1}$). At the peak of magma discharge rate (b) the withdrawal depth (80 m) was greater than that of the upper magma layer and tapping of the lower mafic magma occurred (maximum discharge rate $= 1.4 \times 10^8 \text{ kg s}^{-1}$). As magma discharge rate decreased (c) the draw-up cone of magma receded to a shallower level, and mixing occurred between the mafic and evolved layers (maximum discharge rate $\approx 2 \times 10^7 \text{ kg s}^{-1}$).

As magma discharge rate exceeded $7.7 \times 10^7 \text{ kg s}^{-1}$, the withdrawal depth exceeded 70 m, resulting in withdrawal of magma from the lower layer, as shown by the abrupt compositional change in erupted products. Subsequent withdrawal of the lower layer of primitive magma formed a cone through the upper layer (Fig. 3).

From the dimensions of the 4-km-wide Somma caldera, we infer a diameter of 3 km for the Vesuvius magma reservoir. This indicates an initial volume of 1.5 km^3 of the evolved upper-layer magma, of which 0.5 km^3 remained untapped, outside the draw-up cone at the climax of eruption. As evolved magma was withdrawn during the first part of the eruption, the interface between evolved and mafic magma would have migrated about 150 m upwards in the chamber. If tapping of mafic magma at the white/grey boundary resulted primarily from depletion of the upper evolved layer, it is difficult to explain the increase in the proportion of evolved magma in the late-stage hybrid products (Fig. 1). The link between reversal of chemical trends in the later stages of the eruption and decrease in magma discharge rate point to withdrawal rate as an important factor in controlling the composition of erupted magma as opposed to continued depletion of the upper evolved layer. As the magma discharge rate decreased during eruption of grey pumice, the withdrawal depth was less than the thickness of the shrinking evolved magma layer, leading to increased mixing with the primitive lower layer magma (Fig. 3). We estimate a maximum mixing of 20% of the evolved endmember with the primitive magma in the final stages of the plinian phase.

As shown by this event and also indicated by variable pyroclast size grading patterns in other fallout deposits, magma withdrawal during explosive eruptions is typically not characterized by a constant flux of magma from a crustal chamber¹³. Magma discharge rates during plinian phases can vary by an order of magnitude and are likely to have an even greater range during the transition from plinian fallout to pyroclastic flow¹⁴. In addition, magma discharge rate inferred by pyroclast-dispersal modelling may be different than the evacuation rate from the chamber if significant temporal changes in bulk density occurs in the conduit. This is likely to occur at the start of an eruption before steady-state conditions are established. A further complication in the withdrawal process is the transition from a central vent (plinian phase) to multiple ring vents (pyroclastic flow generation) when large volumes of silicic magma are erupted during caldera formation. Both of these factors are likely to have a profound effect on the disruption of pre-existing compositional gradients in magma chambers. Translation of erupted product gradients back into chamber gradients must therefore

consider effects of eruption rate and vent geometry during magma withdrawal from crustal reservoirs. □

Received 13 November 1989; accepted 12 April 1990.

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ACKNOWLEDGEMENTS. We thank T. Druitt for his review of the manuscript. This work was supported by the National Science Foundation and the National Geographic Society.

High-field-strength element depletions in arc basalts due to mantle–magma interaction

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BASALTS, basaltic andesites and andesites in subduction-related magmatic arcs are all depleted in high-field-strength elements (such as Ti, V, Zr, Nb, Hf and Ta) relative to mid-ocean-ridge basalt (MORB). Here we show that these depletions can be produced in liquids by interaction with depleted mantle peridotite, as would occur during the ascent of an arc magma through the mantle lithosphere of the overriding plate. This process involves extensive reaction between liquid, olivine, orthopyroxene and spinel. High-field-strength element depletions are produced because olivine, orthopyroxene and spinel have higher crystal/liquid distribution coefficients for these elements than for other incompatible trace elements. Liquids modified by mantle–magma interaction will also be depleted in heavy rare-earth elements, Cr and Ni, and enriched in light rare-earth and large-ion lithophile elements, relative to MORB. These characteristics are all common in mafic magmas at convergent plate margins^{1–7}.

Our trace-element modelling complements recent experimental and theoretical investigations, which show that reaction between magnesian olivine tholeiite and depleted peridotite can produce the major-element characteristics of calc-alkaline basalt, basaltic andesite and andesite^{8–12}. Calc-alkaline andesites are found almost exclusively in subduction-related magmatic arcs². Effects of interaction between ascending liquid and upper-mantle wall rock will be enhanced where magma ascends slowly through narrow conduits or by grain-boundary infiltration. The absence of high-field-strength element (HFSE) depletions in most MORBs and back-arc basin basalts is therefore ascribed to relatively limited interaction between liquids and mantle peridotite, due to rapid aggregation and ascent of partial melts at divergent-plate margins.

HFSE depletions have been defined in the following two ways.

(1) Many primitive magmas in subduction-related magmatic arcs have abundances of HFSE, heavy rare-earth elements (HREEs) and some compatible elements (Cr, Ni) that are lower

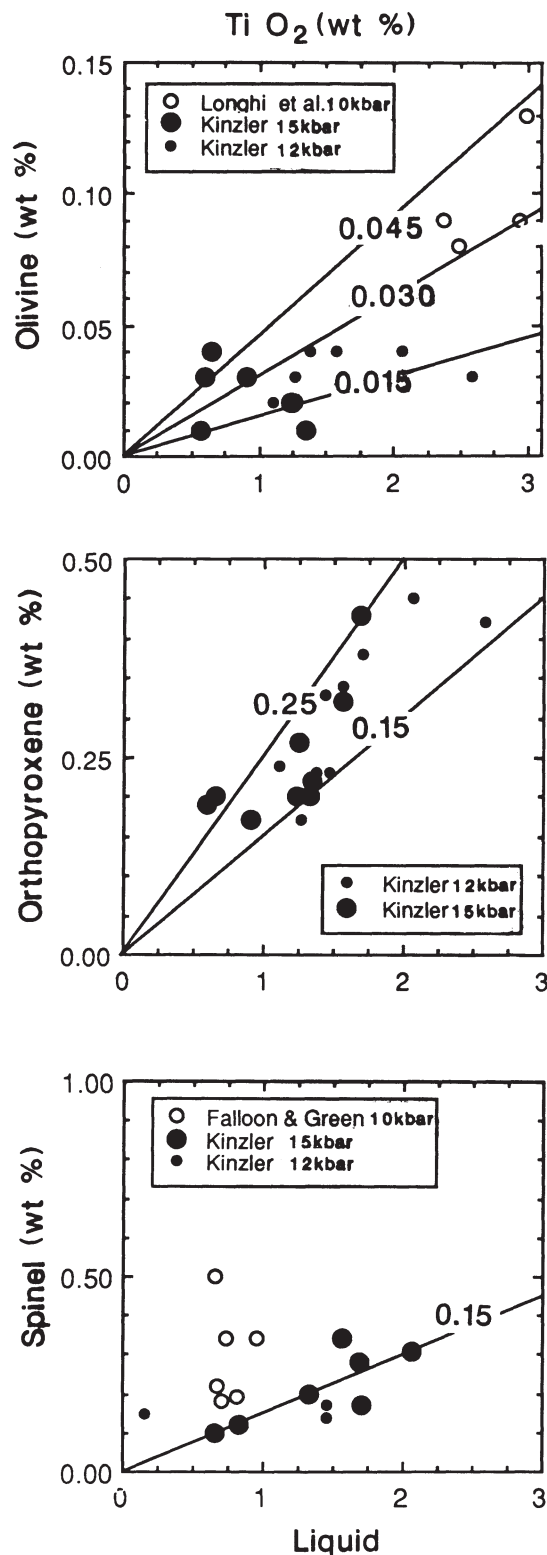


FIG. 1 Electron-microprobe analyses of TiO_2 (weight per cent) in coexisting olivine, orthopyroxene, Cr–Al spinel and liquid (quenched glass) from experiments on magnesian basalt compositions at pressures ≥ 10 kbar. On the basis of these and other data^{22–27} we have adopted values of 0.015 for $D_{\text{liq}}^{\text{ol}}(\text{TiO}_2)$, 0.15 for $D_{\text{liq}}^{\text{opx}}(\text{TiO}_2)$, and 0.15 for $D_{\text{liq}}^{\text{spinel}}(\text{TiO}_2)$ in the upper mantle. Data of Longhi *et al.*²⁴ are for lunar basalts at an oxygen fugacity buffered by Fe–FeO. Data of Falloon and Green³⁷ are for basalt–peridotite mixtures at 10 kbar. Data of Kinzler (this paper and R.J.K. and T. L. Grove, manuscript in preparation) are for MORB + olivine bulk compositions at 12 and 15 kbar. Oxygen fugacity was not buffered in the experiments of ref. 37 and of R.J.K. and T. L. Grove, but all were performed in graphite inner capsules.

than those of primitive MORB. These characteristics have been attributed to the stabilization of a phase with HFSE crystal/liquid distribution coefficients ($D_{\text{liq}}^{\text{xl}}$ = concentration in crystal/concentration in liquid) > 1 in the arc magma source, to metasomatism of the arc magma source by a fluid or magma depleted in HFSE, to a high degree of partial melting, and/or to early fractionation of magnetite^{1-7,13,14}. In general agreement with our study¹⁵, Meen and Ayers¹⁶ attributed HFSE depletions to reaction with mantle wall rock. (2) HFSE anomalies have been defined as depletions or enrichments of HFSEs relative to the adjacent elements on compatibility diagrams. The order of elements on such diagrams has been determined on the basis of smoothness of the normalized trace-element pattern of MORB (ref. 17 and references cited therein). The Zr anomaly is defined as $2\text{Zr}/(\text{Sm} + \text{Nd})$, and Ti anomaly as $2\text{Ti}/(\text{Eu} + \text{Tb})$, using chondrite-normalized concentrations. Where the anomalies are less than unity for HFSEs in a solid or liquid phase, the phase is considered depleted in HFSEs. Depletions defined in this way are common in arc magmas^{4-7,13,14}, and occur in clinopyroxenes from mantle peridotites^{18,19}. Mantle-magma interaction can produce both types of HFSE depletion.

We have used Zr and Ti as analogues for all HFSEs. The crystal/liquid distribution coefficients for other HFSEs in olivine (ol), orthopyroxene (opx) and spinel are virtually unknown. We use generally accepted values of $D_{\text{liq}}^{\text{xl}}$ for rare-earth elements (REEs), Zr and Ti, supplemented by new experimental data for $D_{\text{liq}}^{\text{spinel}}$, $D_{\text{liq}}^{\text{opx}}$ and $D_{\text{liq}}^{\text{ol}}$. There is considerable uncertainty in these

values owing to inhomogeneity of products in experiments, analytical errors arising from low concentrations, and incomplete characterization of variation of $D_{\text{liq}}^{\text{xl}}$ with temperature, pressure and bulk composition. It is especially worrisome that $D_{\text{liq}}^{\text{xl}}$ for REEs, Ti and Zr have rarely been measured in the same experiment. Here we adopt the lowest $D_{\text{liq}}^{\text{xl}}$ for Ti and Zr compatible with experimental data. These minimize fractionation of HFSEs by reaction; larger values would produce larger HFSE depletions. An essential point is the adoption of $D_{\text{liq}}^{\text{ol}}$ for REEs determined by McKay²⁰. Slightly higher values from the algorithm of Colson *et al.*²¹ could be used, but the algorithm does not fit McKay's data for light rare-earth elements (LREEs). We consider much higher values of $D(\text{REE})_{\text{liq}}^{\text{ol}}$ to be incorrect, in agreement with McKay²⁰. Aside from this, the qualitative results of our modelling can be reproduced using virtually any consistent set of $D_{\text{liq}}^{\text{xl}}$ from the literature.

Published $D_{\text{liq}}^{\text{xl}}$ for Zr and Ti in olivine and orthopyroxene²⁰⁻²⁷, and new results presented here (Fig. 1) for Ti in olivine, orthopyroxene and Cr-Al spinel, show that these two HFSEs have $D_{\text{liq}}^{\text{xl}}$ s that are orders of magnitude larger than $D_{\text{liq}}^{\text{xl}}$ s for REEs in these phases (Table 1 and references cited therein). Zr and Ti are not strongly fractionated from the REEs in liquids produced by partial melting (Fig. 2a). But during mantle-magma interaction, liquid may react with large masses of olivine, or orthopyroxene and spinel, and these phases will fractionate Zr and Ti relative to REEs (Fig. 2b-d).

We modelled mantle-magma interaction using the equations in refs 8 and 28 for combined assimilation and crystal fractionation. Results are presented in Fig. 2. The reactants used are depleted-mantle lherzolite (Fig. 2 and Table 1), and aggregate liquid from 3% melting of pyrolite²⁹ (Fig. 2). Liquids formed by partial melting of four-phase peridotite will become saturated only in olivine ($\pm$ Cr-rich spinel) on decompression³⁰⁻³². Their reaction with lherzolite will involve incongruent dissolution of pyroxene to produce olivine + spinel + modified liquid. Although decompression alone should increase the mass of liquid in mantle-magma systems, we suggest that this effect may be balanced in natural systems by cooling and chemical effects of wall-rock reaction⁸⁻¹⁰ during ascent. For example, possible increase in magma mass owing to pyroxene dissolution would decrease the water content of initially hydrous liquids, leading to increased crystallization. For these reasons, and for simplicity, we model mantle-magma interaction with constant magma mass (mass assimilated/mass crystallized, $M_a/M_c = 1$).

Three different types of reaction between basaltic magma and lherzolite are illustrated. (1) In Fig. 2a dissolution of pyroxene is small, so phase proportions in the lherzolite remain constant. (2) In Fig. 2b ascending magma dissolves some pyroxene, but not all, leaving a harzburgite residuum. (3) In Fig. 2c all pyroxene in the wall rock dissolves, leaving a dunite residuum. Liquid products of reactions involving extensive pyroxene dissolution will probably be calc-alkaline basalt, basaltic andesite and andesite; derivative liquids of reactions involving minimal pyroxene dissolution will generally be tholeiitic⁸⁻¹⁰. All these reactions produce depletions of Zr and Ti in derivative liquids. Reactions (1) and (2) also lead to depletion of REEs relative to MORB. If liquid mass decreases slightly ($M_a/M_c = 0.9990$), owing to reaction stoichiometry and/or cooling of ascending magma, Cr and Ni are also depleted.

Many of the distinctive trace-element characteristics of primitive arc magmas can be accounted for by reactions in which liquids formed by partial melting of fertile peridotite at depth interact with depleted upper-mantle wall rocks during ascent. During the formation of ocean crust, the uppermost mantle is subjected to the largest degree of melting, with progressively deeper lherzolites undergoing progressively less depletion in basaltic components. We envision an initial state in which such variably depleted mantle becomes part of the overthrust plate in a subduction zone. Melting of a fertile source produces liquids that rise through, and react extensively with, more-depleted

TABLE 1 Phase proportions and distribution coefficients used in modelling

(a) Phase proportions (weight per cent)					
	Pyrolite ²⁹	Melting mode*	lherzolite (residue of 16.5% melting of pyrolite)	Harzburgite (50% lherzolite 50% dunite)	Dunite
Olivine	55.2	-20	69	83	97
Orthopyroxene	24.7	25	24	12	0
Clinopyroxene	17.8	83	5	2.5	0
Spinel	2.3	2	2	2.5†	3†

(b) Crystal/liquid distribution coefficients at ~10 kbar (by weight)				
	Olivine‡	Orthopyroxene§	Clinopyroxene	Spinel¶
La	0.000007	0.0025	0.04	0.0006
Ce	0.00001	0.005	0.13	0.0006
Nd	0.00007	0.01	0.25	0.0006
Zr	0.007	0.07	0.20	0.07
Sm	0.0007	0.02	0.45	0.0006
Eu	0.00095	0.03	0.50	0.0006
Gd	0.0012	0.04	0.50	0.0006
Ti	0.015	0.15	0.30	0.15
Dy	0.004	0.05	0.51	0.0015
Er	0.009	0.07	0.52	0.003
Yb	0.023	0.11	0.52	0.0045
Cr	2.0	8.0	2.0	200.0
Ni	11.0	3.5	3.0	10.0

* The melting mode was determined by solution of the matrix $\text{X} \cdot \text{ol} + \text{X} \cdot \text{opx} + \text{X} \cdot \text{cpx} + \text{X} \cdot \text{spinel} = 100 \cdot \text{liq}$ using data on coexisting liquid and solid phases in spinel lherzolite at 10–20 kbar, adjusted to produce approximately constant modal proportion of spinel during melting.

† The modal proportion of spinel produced by pyroxene dissolution is calculated assuming that virtually all Cr in pyroxene reactants consumed is incorporated in spinel, pyroxenes contain ~1 wt% Cr_2O_3 , and the proportion of Cr_2O_3 in spinel is approximately constant at 30 wt%.

‡ REEs, ref. 20; Zr and Ti, refs 23–27, this paper, P.B.K., unpublished ion-probe data, R. J. K. and T. L. Grove, manuscript in preparation; Cr and Ni, ref. 23 and references cited therein.

§ REEs, ref. 35, clinopyroxene partition coefficients; Zr and Ti, ref. 22, this paper, R. J. K. and T. L. Grove, manuscript in preparation; Cr and Ni, ref. 23 and references cited therein.

|| REEs, ref. 36, K. T. M. J. and R. J. K., unpublished ion-probe data; Zr and Ti, ref. 23 and references cited therein, refs 25, 26, 36, K.T.M.J. and R.J.K., unpublished ion-probe data; Cr and Ni, ref. 23 and references cited therein.

¶ REEs, ref. 35, clinopyroxene partition coefficients; Zr, by analogy to clinopyroxene with $2D(\text{Zr}) \geq D(\text{Ti})$; Ti, this paper, R. J. K. and T. L. Grove, manuscript in preparation; Cr and Ni, ref. 23 and references cited therein.

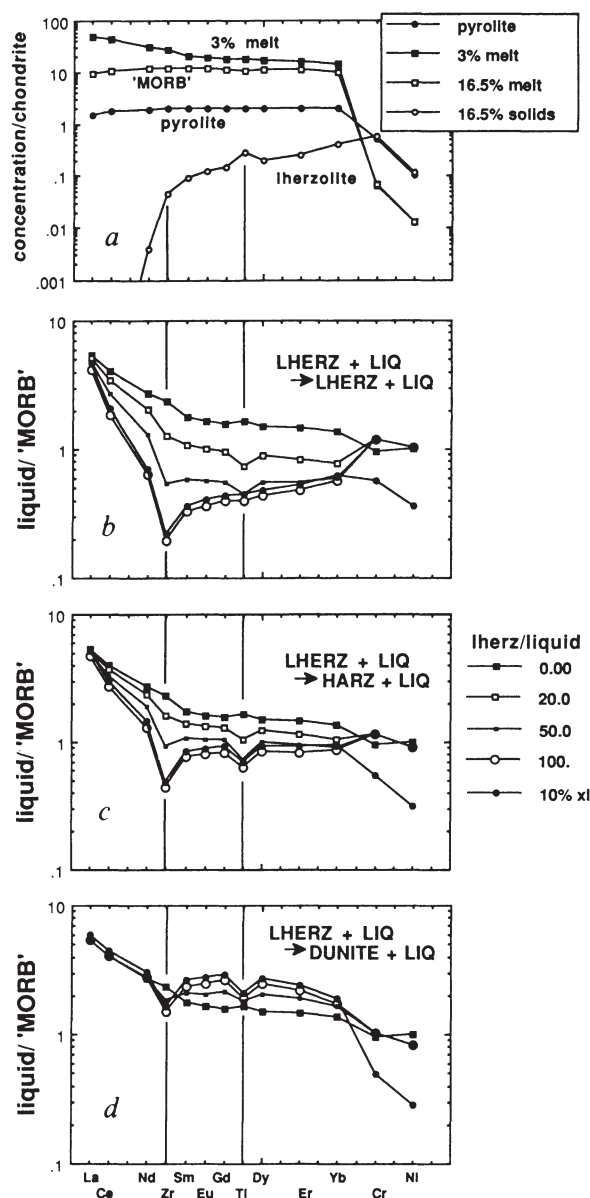


FIG. 2 Results of modelling the reaction between basaltic magma and depleted-mantle lherzolite. Phase assemblages, bulk compositions and D_{liq}^{xl} s given in Table 1. *a*, Initial solid and liquid compositions used in modelling. Initial compositions produced by fractional melting of pyrolite²⁹. Fractional melting does not significantly fractionate HFSEs from REEs in aggregate liquids. This is true for liquids produced by batch melting (not shown) as well. Curve labelled 3% is aggregate liquid from 3% fractional fusion. MORB is putative mid-ocean-ridge-basalt composition, and is the aggregate liquid formed by 16.5% fractional fusion. Lherzolite is the bulk-solid composition after 16.5% fractional fusion, and is similar to abyssal peridotites, which have strongly depleted REE patterns¹⁹. Initial pyrolite composition has REEs from 1.5 (for La) to 2 (for Sm to Yb) times the chondritic abundance, Zr and Ti = $2 \times$ (chondritic), Cr = $0.5 \times$ (chondritic), and Ni = $0.1 \times$ (chondritic)^{38,39}. Order of elements as for compatibility diagrams of ref. 17 and references cited therein. *b*, *c*, *d*, Results of reaction between basalt and depleted lherzolite: Wall-rock reaction in the upper mantle produces HFSE depletion, accompanied by HREE depletion relative to MORB, in derivative liquids. Initial liquid (mass lherz/mass liquid = 0.00) is aggregate melt from 3% fractional melting of pyrolite. Lherzolite reactant is residue of 16.5% fractional melting of pyrolite. Reaction modelled using equations of refs 29 and 8. Key to abbreviations: 'MORB' = aggregate melt produced by 16.5% fractional melting of pyrolite; LHERZ, HARZ, DUNITE denote lherzolite, harzburgite and dunite phase assemblages, respectively; LIQ, liquid; lherz/liquid, mass of lherzolite reactant to mass initial liquid in the system. All calculations for constant magma mass except curves labelled 10% xl. In these, magma mass decreases 10% during reaction, mass lherz reactant to mass liquid = 100, and $M_0/M_c = 0.9990$.

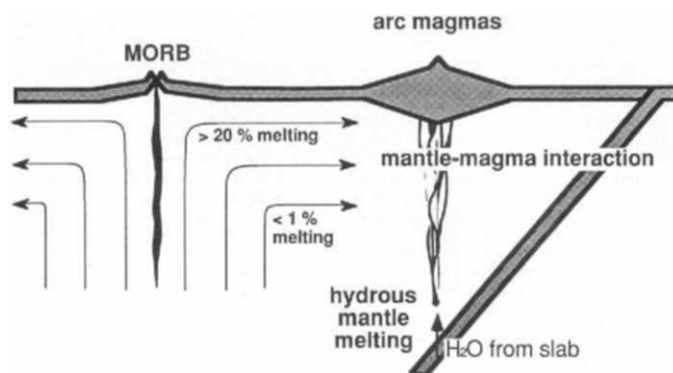


FIG. 3 Schematic illustration of mantle melting processes. At spreading centres, the aggregate melts of large degrees of fractional melting rise to form MORB. In subduction zones, H_2O from the downgoing slab rises into the mantle and causes small degrees of partial melting in fertile mantle at depth. Liquids generated here pass slowly upward through, and interact with, depleted peridotite wall rock.

mantle lithosphere on their way to form new arc crust (Fig. 3). Both liquid and solid products of reaction will develop HFSE and HREE depletion during this process. This hypothesis is consistent with the theory that some calc-alkaline basalts, basaltic andesites and andesites are produced by reaction between ascending tholeiitic magmas and mantle wall rock⁸⁻¹⁰.

We believe that the following results are general: reaction will produce depletions relative to MORB in Zr, Ti and REEs in derivative liquids. Where initial liquids are enriched in REEs and large-ion lithophiles relative to MORB, this enrichment will be retained in derivative liquids. Where magma mass decreases slightly during reaction, concentration of Cr and Ni in derivative liquids will be lower than in primitive MORB. Although reaction will produce large variations in trace-element concentration, $Mg/(Mg+Fe)$ will be high and approximately constant in derivative liquids⁸⁻¹⁰.

Our calculations do not require ascending liquid to reach major-element or trace-element equilibrium with surrounding mantle wall rock. Calculated mass ratios of reactant lherzolite to initial liquid are similar to fluid/rock ratios in metamorphic petrology. They represent the minimum mass of lherzolite necessary to produce a given liquid composition, assuming complete equilibrium between reactants is attained. Our results would be qualitatively unchanged even if some reaction mechanisms, such as dissolution of pyroxenes, were more rapid than others, such as solid diffusion in olivine and spinel. Our results are, however, dependent on the implicit assumption that rates of exchange reactions for Zr and Ti are greater than or equal to rates of exchange reactions for REEs, on the timescale of mantle-magma interaction.

Isotope analyses of many arc magmas indicate the presence of a slab-derived component in their source (refs 1, 2, 33, 34 and references cited therein). But the slab-derived component need not have the trace-element characteristics, such as HFSE depletion, which distinguish arc basalts from MORBs. Although other hypotheses are viable, we believe that the essential contribution of the slab-derived component in arc magmatism is water, which leads to partial melting in the overlying mantle wedge. HFSE depletions arise as partial melts formed in the wedge move slowly up and react with mantle wall rock. □

Received 20 February; accepted 11 April 1990.

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ACKNOWLEDGEMENTS. We thank R. Arculus, J. Davidson, H. Dick, F. Frey, J. Gill, V. Salters, N. Shimizu, T. Sisson and B. Wasson for comments on the manuscript. This work was supported in part by the Woods Hole Oceanographic Institution.

Induced vertical migration in copepods as a defence against invertebrate predation

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THE adaptive significance of diel vertical migration by planktonic organisms is often explained in terms of daily changes in foraging locations that reduce the risk of depth-stratified predation^{1–3}. The timing, extent and rate of migration are usually presumed to be more or less invariant (closed⁴) behaviours that have evolved under local predation regimes^{5–8}. Observational and experimental evidence, however, indicate that migratory patterns in the presence of predators may be altered substantially by short-term reduction in the availability of food^{9–13}. Behavioural flexibility of food-limited individuals rather than genetic change of populations seems likely¹³. Whether individuals are also sensitive to changes in the risk of predation is unknown, though some field observations are highly suggestive¹⁴. Here I report rapid initiation (<4 h) of vertical migration in previously non-migrating freshwater copepods inside large *in situ* enclosures on exposure to an invertebrate predator. Responses of copepods to water that had previously held captive predators suggests that a chemical cue is involved. These results show that crustacean zooplankton may be capable of flexible, predator-sensitive foraging and suggest a mechanism for rapid changes in migration patterns.

Larval phantom midges *Chaoborus* spp. (Diptera; Chaoboridae) are large, 'nocturnal'¹⁴ vertical migrators (night-time ascent, daytime descent) that eat other freshwater zooplankton. Some prey taxa abandon more productive shallow strata during nocturnal incursions of chaoborids and establish opposing 'reverse' vertical migrations^{15,16} (night-time descent, daytime ascent). I examined a reverse migration by the calanoid copepod *Diaptomus kenai* in oligotrophic Gwendoline Lake (49°19' N, 122°34' W, 13 ha, 27 m maximum depth, mean Secchi

depth 8 m) before and after chaoborids were eliminated by escaped juvenile trout (*Salmo clarki*). Predatory mortality of copepods (annually ~20% (refs 16, 17)) became negligible for the next 3 yr (no invertebrate predators of copepods detected in 166 bi-weekly plankton samples and only 9 *D. kenai* in 27,128 diet items from 88 netted and angled trout) as trout focused on abundant alternative prey.

When re-examined 28 months (four copepod generations) after midge extermination, the enhanced population of *D. kenai* had ceased migrations (Fig. 1). All copepods continuously occupied more productive shallow waters previously exploited only during daylight hours. Grazable algal concentrations were 30–50% higher than in pre-fish samples (Fig. 1), presumably as a result of reduced cladoceran grazing under fish predation. Whether migratory behaviour had been lost through natural selection for non-migratory morphs or through phenotypic plasticity of individuals, and whether increased abundance of phytoplankton had any measurable consequences on altered migration were examined in experimental enclosures.

I exposed *D. kenai* from Gwendoline Lake, which had been free of phantom midge predation for four copepod generations, to imported *Chaoborus trivittatus* inside 1.5 m × 15 m deep polyethylene tubes inserted in Gwendoline Lake. Migratory fourth instar midge larvae (2,500 per enclosure, collected in a nearby fishless lake) and Gwendoline Lake zooplankton were stocked at historical pre-fish densities. No fish were stocked in any enclosures. Triplicate 100-litre, depth-stratified pump samples¹⁷ taken at 1.5 m intervals were used to estimate plankton densities and document vertical spatial distributions. Sampling began 4 h after adding crustaceans and chaoborids to enclosures that had been filled with lake water earlier that day.

Initial samples taken after dark revealed that exposure to chaoborids had rapidly (<4 h) stimulated a reappearance of reverse migrations in these previously non-migratory individuals (Fig. 2). Copepods descended to the bottom of all four predator enclosures and remained there until dawn. Not one copepod was detected in surface waters after dark, where added *Chaoborus* larvae were abundant. Copepods in controls (added lake water, no chaoborids) remained at shallow depths after dark, as did copepods in the *Chaoborus*-free lake (Fig. 1). Ascent on the following morning required their passing through the mass of descending *Chaoborus* larvae, indicating that reverse vertical migration had been re-adopted, and that the downward

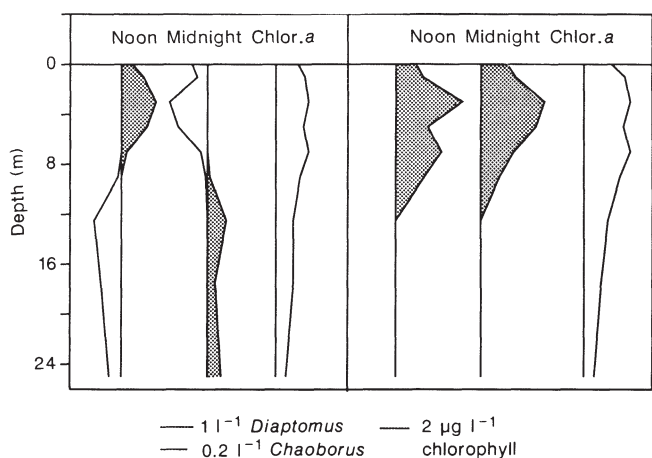


FIG. 1 Mean vertical distributions of the copepod *Diaptomus kenai* (stippled) and the midge predator *Chaoborus trivittatus* (open) in Gwendoline Lake between 1100 and 1330 h (noon) and 2330 and 0200 h (midnight) on four dates during the month of July. Samples were taken by Clark-Bumpus sampler at 2-m depth intervals. Vertical distributions of chlorophyll *a* (pump samples at 0.5 m intervals) are for 5–50- μ m fraction collected during the day. Each figure is a mean of 12 samples (3 replicates on 4 dates) and is taken to represent the summer mean. Left-hand panel, vertical profiles 20 months before *Chaoborus* extermination by trout. Right-hand panel, vertical profiles 28 months after *Chaoborus* extermination.

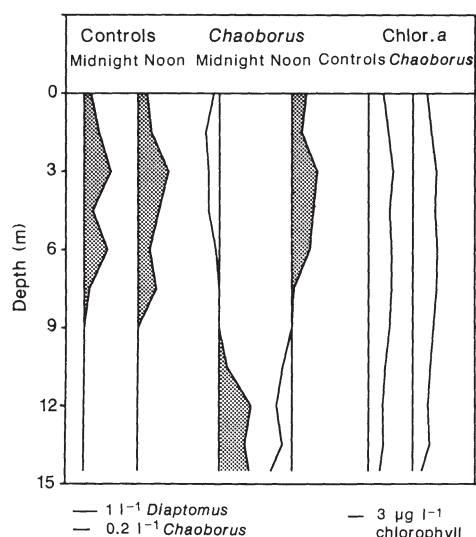


FIG. 2 Mean noon and midnight depth distributions of enclosed copepods, fourth instar *Chaoborus* larvae, and 5–50- μ m fraction chlorophyll *a* in 1.5 m $\times$ 15 m tubes. Controls contained no *Chaoborus*. Enclosures were sampled at the same time as the lake (right-hand panel, Fig. 1). Zooplankton were stocked at historical pre-fish densities 4 h before midnight sampling. Noon samples were taken the following day, $\sim$ 16 h after stocking. Chlorophyll measurements were taken during the noon sampling period.

movement was not merely predator avoidance. Opposing patterns of diel migration of *D. kenai* and *C. trivittatus* continued unabated for 5 weeks in all four treatment replicates before enclosures were dismantled. Copepods from the four controls and the lake were non-migratory, remaining at shallow depths during this time.

No systematic differences were detected between control and predator enclosures in profiles of temperature–depth, total chlorophyll *a* and 5–50- μ m size-fractionated chlorophyll *a* (Fig. 2), in epilimnetic or hypolimnetic ‘grazable’ phytoplankton counts, or in the depth profiles of any of the six other crustacean taxa in the enclosures. Thus, differences in food concentrations between predator and control enclosures are unlikely to have caused the sudden change in copepod migration pattern. Rather, exposure to *Chaoborus trivittatus* apparently stimulated

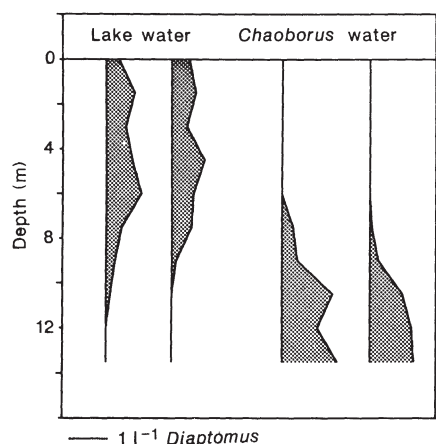


FIG. 3 Night-time vertical distributions in enclosures of previously unexposed *D. kenai* after addition of 20 litres of 25 μ m filtered lake water that did or did not previously contain fourth instar *C. trivittatus* larvae ($\sim$ 110 per litre for 24 h). Two replicates are presented for each experiment. Test water was added slowly to the 2–6-m depth stratum of each enclosure through a tube and copepod distributions were sampled in darkness 4 h later.

TABLE 1 Characteristics of vertical migration by *Diaptomus kenai* from Gwendoline Lake when exposed to *Chaoborus trivittatus*

	Pre-fish	Post-fish	<i>P</i>
Median midnight depth (m)	12.2	12.9	>0.4
(range of medians)	(11.4–12.9)	(11.6–13.4)	
Median noon depth (m)	3.7	4.0	>0.6
(range of medians)	(3.4–4.4)	(3.7–4.6)	
Time after local sunset to reach median night-time depth (min)	47	42	
Time before local sunrise to reach median daytime depth (min)	43	53	
Rate of descent (m min^{-1})	1.04	1.19	>0.2
(s.e.)	(0.20)	(0.21)	
Chlorophyll <i>a</i> ($\mu\text{g l}^{-1}$)	1.76	2.87	<0.001
at 4 m depth (s.e.)	(0.17)	(0.11)	

expression of latent migration in individual *Diaptomus kenai*. Further, timing, extent and rate of this migration differed in no way from that measured 5 yr previously in enclosures containing chaoborids when chlorophyll *a* concentrations were $\sim$ 40% lower (Table 1). Thus, within the range of 1.5–2.9 $\mu\text{g l}^{-1}$ chlorophyll *a*, migratory behaviour in these copepods was insensitive to food concentration, unlike *Daphnia* studied elsewhere¹³. Anti-predator migration in long-lived copepods adapted to oligotrophy may be more closed to modification by food supply than it is in more *r*-selected cladocerans³. Alternatively, vertical migration may be more noticeably affected by extreme food limitation.

Rapid induction of migratory behaviour in every detected individual exposed to the predator suggests persistence of the genes for migration at high frequency despite four generations without measurable predation. It is possible that genetic fixation had occurred before *Chaoborus* extermination. But the low maintenance cost of the trait in an unexpressed state probably also favoured its preservation¹⁸. The capacity to respond flexibly to large variations in predator recruitment¹⁹ would seem to provide strong selective value to a long-lived copepod.

A chemical cue may be involved in copepod detection of the presence of chaoborids. I induced vertical migration experimentally in naive copepods by exposure to water that had previously held midge larvae. Twenty litres of sieved (25 μ m) lakewater from which larvae had been carefully netted after being held for 24 h (110 larvae per litre) were gently added to the 2–6-m depth stratum containing predator-naive crustaceans in two 15-m deep enclosures. Two controls received similarly treated lake water lacking *Chaoborus*. Four hours later, night sampling of *D. kenai* determined that, in enclosures receiving *Chaoborus* water, almost 90% of copepods were in the bottom half of the enclosure; in control enclosures, on the other hand, 90% were in the upper half (Fig. 3). Previously unexposed copepods apparently detected the predator from some material passing through the 25 μ m sieve.

Inducible morphological defences against predators are now known in several invertebrate groups^{20–25}. Chemical induction is known in rotifers²³ and cladocerans^{23,24} among zooplankton, though not in copepods. In contrast, induction of vertical migration under variable risk of predation has not been shown previously for invertebrate plankton²¹ at natural depth scales. Correlations between depth distributions of marine copepods and seasonally changing invertebrate predator densities¹⁴, however, suggests that other copepods may also display predator-sensitive use of vertical space. The role of changing food availability in these observations however is unknown. In oligotrophic Gwendoline Lake, food concentration played no apparent part in altering sensitivity of individual zooplankton to predators. □

Received November 16 1989; accepted 15 March 1990.

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ACKNOWLEDGEMENTS. I thank Alan Redenbach, Robert Newsome and Danuta Dolecki for technical assistance. This work was supported in part by the Natural Sciences and Engineering Research Council of Canada.

Compounds in the droplets of the orb spider's viscid spiral

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THE orb web of the garden spider *Araneus diadematus*, like those of other ecribellate orb spiders, relies on the remarkable extensibility of its sticky capture spiral to intercept and entrap prey^{1,2}. The spiral strands consist of core fibres and an all-enveloping aqueous coat, which forms into a pattern of droplets³. The core fibres are paired and probably made of typical spider silk^{4,5}. The aqueous solution droplets are more or less evenly

spaced⁶ and are required to allow the special mode of extension of the fibres^{3,7}. Knowledge of the chemical composition and general physical properties of this liquid phase is necessary to understand its function in web mechanics and prey capture⁸. We have now investigated the chemical composition of this aqueous solution and found the droplets to be a concentrated solution of hygroscopic substances related to neurotransmitters. We offer an explanation as to the function and origin of this solution.

To determine the chemical composition of the aqueous solution, we examined the wash of several entire webs of *Araneus diadematus*, *Araneus cavaticus*, *Argiope aurantia* and *Argiope trifasciata* by NMR procedures. Webs were washed in water and/or chloroform, and sometimes the water wash was further fractionated into ethanol-soluble and -insoluble compounds⁹. We found (Fig. 1) GABamide (γ -aminobutyramide), *N*-acetyltaurine, choline, betaine and isethionic acid to be present in a surprisingly strong solution (Table 1). Previously only GABamide has been identified in the webs of *Argiope* (Araneidae)^{10,11} and *Latrodectus* (Theridiidae)¹², although it has been suspected in the web of *Araneus*¹³, as has *N*-acetyltaurine in *Argiope*¹⁰.

Other substances previously reported in aqueous web washes were: cysteic acid (2%)¹³, lysine (2%)¹³, serine (2%)¹³, potassium nitrate (7%)¹⁴, potassium dihydrogenphosphate (3%)¹⁴, and

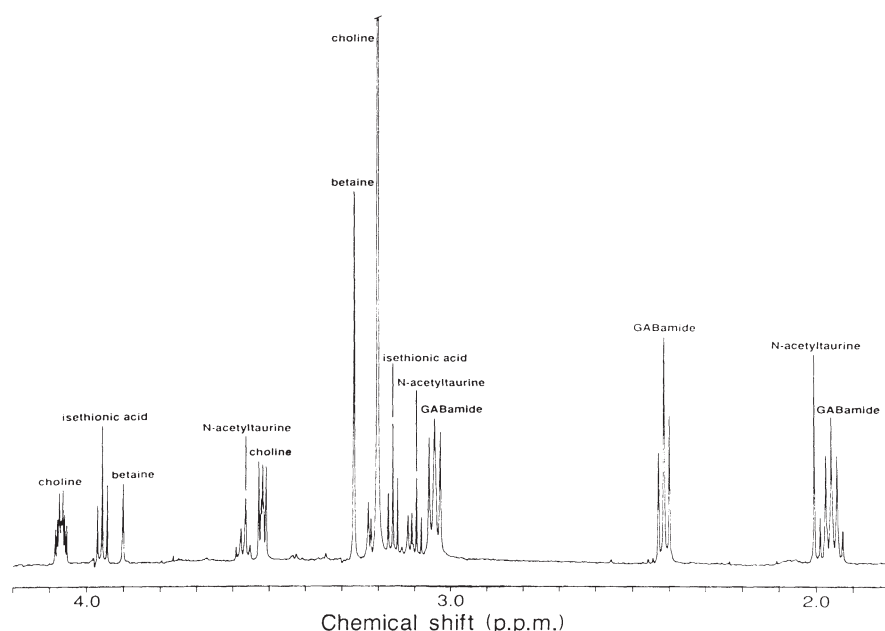


FIG. 1 NMR spectrum of the water-soluble fraction obtained from 11 *Araneus diadematus* webs. Spectra were recorded using a Bruker AM500 spectrometer. The solution contained 20% D₂O. The H₂O peak was suppressed by gated irradiation. TSS was used as an internal standard at 0 parts per million. Spin-spin correlations were determined from COSY spectrum³³. Unambiguous assignment of resonances was achieved by addition of the respective compounds to the solution. The 500-MHz 1H-NMR spectra of choline, betaine, isethionic acid, *N*-acetyltaurine and GABamide were found to be coincident with the peaks labelled in the spectrum. The spectrum also contains peaks (not shown) due to unidentified small branched-chain aliphatic compounds (possibly pheromones) with intensities <10% of the major identified peaks. The concentrations of the major constituents were determined from the integrated areas of the peaks relative to a known concentration of TSS in 1 sample from 11 webs and 2 samples from 7 webs. Calculation of the concentration in the droplets was based on an estimation of 150,000 droplets per web with a volume of 5×10^{-6} mm³ per droplet (see Table 1). All web material was collected by glass rod from new webs built by mostly immature *Araneus diadematus* in clean plastic boxes in the laboratory. The material was suspended for several days at 4 °C in distilled water or pure chloroform and occasionally agitated. The material was weighed before and after washing (and drying in an oven at 80 °C).

TABLE 1 Concentrations of the major water-soluble components of the web

		A.d.	A.c.	A.a.	A.t.
Choline	$(\text{CH}_3)_3\text{NCH}_2\text{CH}_2\text{OH}$	1.5 M	12%	20%	16%
Betaine	$(\text{CH}_3)_3\text{NCH}_2\text{COO}^-$	0.2 M	2%	3%	3%
Isethionic acid	$\text{HOCH}_2\text{CH}_2\text{SO}_3^-$	1.1 M	23%	6%	21%
N-acetyltaurine	$\text{CH}_3\text{CONHCH}_2\text{CH}_2\text{SO}_3^-$	0.3 M	6%	17%	5%
GABamide	$^-\text{NH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CONH}_2$	1.9 M	45%	48%	6%

For *Araneus diadematus* (A.d.) the concentrations are given in moles. Here the concentration of GABamide varied by a factor of around three between different samples. The concentrations of the remaining compounds varied by less than a factor of two. In *Araneus cavaticus* (A.c.), *Argiope aurantia* (A.a.) and *Argiope trifasciata* (A.t.) the concentrations are given as mole-percent of the ethanol extract. Not all compounds present are accounted for in the table.

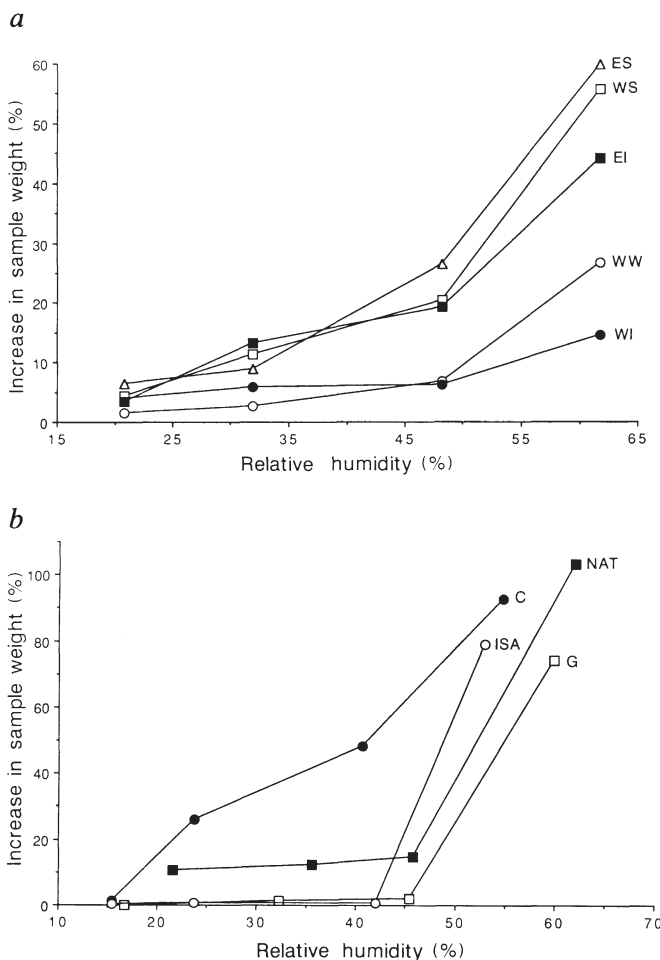


FIG. 2 *a*, Water uptake of web fractions and constituent compounds. Water uptake by *Argiope aurantia* webs and web fractions. WW, whole web (○); WI, water-insoluble fraction (●); WS, water-soluble fraction (□); EI, ethanol-insoluble subfraction of WS (■); ES, ethanol-soluble subfraction of WS (△). WW=100, WI=43.9, EI=9.6 and ES=31.2% of the whole web weight as determined gravimetrically after each fractionation step. *b*, Hygroscopicity of standard compounds found in the aqueous wash of webs. Each data point is the mean of three measurements. NAT, N-acetyltaurine (■); C, choline chloride (●); G, GABamide (□); ISA, sodium isethionate (○). KNO_3 , KH_2PO_4 , glycine and betaine HCl each gained less than 3% at relative humidity (R.H.) up to 55%.

METHODS. Whole webs were collected under laboratory conditions, pooled and fractionated with water and ethanol as previously described⁹. Whole webs or web fractions were brought to constant weight *in vacuo* over P_2O_5 . These were placed individually in a sealed chamber of desired R.H. using the appropriate salt solution^{35,36}. The absorption of water was monitored continuously with a Cahn 2000 microbalance until no further weight gain was observed (*a* and *b*). The R.H. experienced by the sample was then confirmed by placing the probe of a Fischer digital hygrometer above the salt solution at a standard height. All measurements were made at 18–22 °C.

pyrrolidone (8%)¹⁴ (shown to be misidentified GABamide¹¹). Glycoproteins have also been identified in both web extract¹⁵ and spinning gland¹⁶. We did not detect any polymers in our wash but confirmed the presence of KH_2PO_4 and KNO_3 as well as finding glycine and highly saturated fatty acids.

We assume that the high concentrations of the new compounds are present to stabilize the high number of droplets^{17,18}. The presence of so much 'salt' on the fibres dictates the amount of water that the system can take up. The high concentration of the molecules in the droplets gives vapour pressures very close to the ambient humidities commonly met by these spiders. In measurements of water uptake to estimate the contribution of the identified compounds to droplet hygroscopicity (Fig. 2), only choline chloride and N-acetyltaurine showed water absorption over a wide range of relative humidities, comparable to the web itself.

The compounds identified are identical or closely related to compounds known from eubacteria, plants and animals under osmotic stress^{19–24}. To our knowledge, this is the first instance in which such compounds have been observed external to an organism to function under potentially desiccating conditions. We also note that the major components of the solutions are positively charged organic amines, zwitterions or anions of very strong acids—sulphonates. Such compounds are excellent choices for maintaining a strong solution of a given vapour pressure without risking electrostatic interaction with the anionic polymer in the droplets. Moreover, they will not crystallize over a wide range of vapour pressures, thus differing from simple inorganic salts such as NaCl.

Some of the components we found are biochemically related to important neurotransmitters. Functionally, the aggregate glands, which produce the coating liquid, stand out among the seven types of silk glands in *Araneus*. The other silk glands produce threads of traditional, dry silk^{5,6}, which is the ancestral state of spider silk^{25–28}. Phylogenetically, we must assume that the ancestors that first wetted their silk with chemicals used a production mechanism already present in their abdomen. Anatomically, the systems for the local production of synaptic vesicles were available for this purpose^{29,30} as silk glands are typically innervated^{31–32}.

We conclude that, given the design of the remarkably extendable thread of this spider, there is an absolute need for a large number of droplets on the thread to achieve the peculiar properties. The composition of the solution is as good as any that can be devised to prevent evaporation and yet not interact with the fibres, thus allowing the 'windlass' action¹. □

Received 8 January; accepted 28 March 1990.

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ACKNOWLEDGEMENTS. This work was performed independently and in parallel in Durham, New Hampshire, USA (supported by the NRSF and the University of New Hampshire) and in Oxford, UK. We thank D. Edmonds for discussions.

Phototropism and geotropism in maize coleoptiles are spatially correlated with increases in cytosolic free calcium

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PHOTOTROPISM and gravitropism in the shoots and roots of higher plants are the result of asymmetric growth. This is explained by the redistribution of growth regulators following exposure to gravity or unilateral light (the Cholodny–Went hypothesis¹). The positive phototropism² and the negative geotropism of grass seedling coleoptiles are believed to result from lateral movement of auxin from the irradiated to the shaded side^{3–5} and from the upper to the lower side^{6–8}, respectively. Many physiological processes in plants, including auxin-induced cell elongation, are reported to be under the control of calcium^{9,10}. Added auxin triggers oscillations in cytosolic free calcium ($[Ca^{2+}]_{cyt}$) and cytosolic pH (pH_{cyt}) in

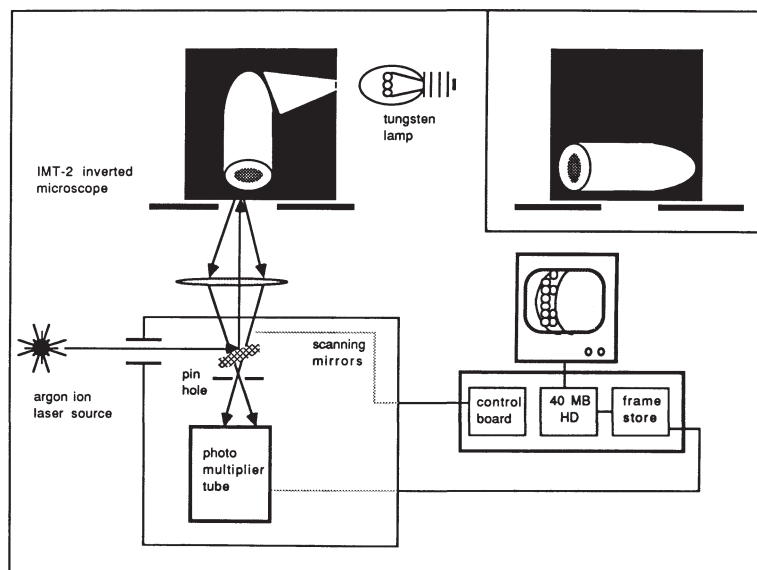
epidermal cells of maize coleoptiles^{11–13}. Until recently¹⁴, it has not been possible to visualize these changes spatially with the commonly used fluorescent cation indicators. Using a scanning laser confocal microscope, a new visible wavelength Ca^{2+} probe fluo-3 and the fluorescent pH indicator BCECF, we have recorded rapid light-induced increases in $[Ca^{2+}]_{cyt}$ and a lowering of pH_{cyt} of cells on the shaded side of maize coleoptiles. In horizontally orientated coleoptiles, $[Ca^{2+}]_{cyt}$ increases and pH_{cyt} decreases in the more rapidly elongating cells on the lower side. For the first time, rapid changes in $[Ca^{2+}]_{cyt}$ and pH_{cyt} are correlated directly with increases in cell elongation stimulated by light and gravity.

Coleoptile tips were loaded with fluo-3 or BCECF (as described in Fig. 1) and viewed with the confocal microscope. The experimental system is diagrammed in Fig. 1.

When the tips of coleoptiles loaded with fluo-3 were irradiated from one side with a tungsten light source (see Fig. 1), substantial increases in Ca^{2+} -dependent fluorescence occurred only in cells on the shaded side (Fig. 2). Increases in fluorescence in the epidermal and peripheral cortical regions (Fig. 2) are consistent with a localized $[Ca^{2+}]_{cyt}$ increase from an initial level of 255 nM (Fig. 2a) to a stabilized level of 415 nM after 15 min of unilateral illumination (Fig. 2d). After the coleoptile was turned through 180°, Ca^{2+} increased from 95 to 215 nM on the shaded (previously illuminated) side and diminished (415 to 305 nM) on the currently illuminated side over a period of 15 min. Such changes in fluorescence were noticeable in both epidermal and

FIG. 1 Schematic diagram of the experimental system used to acquire and process images of fluorescence in coleoptile tips during photo- and gravitropism. Apical coleoptiles loaded with fluo-3 or BCECF were placed on their base on a glass coverslip (thickness 0.017 mm), bathed in tap water (Ca^{2+} , 40 μ M), and viewed on an Olympus IMT-2 inverted microscope with a Nikon CF-Fluor ($\times 10$, n.a. 0.5) objective. The coleoptile chamber was enclosed in a light-tight box and illuminated when necessary through a lateral aperture (5 $\times$ 5 mm) by a tungsten light source (TL intensity: 650 microeinstein $m^{-2} s^{-1}$). The fluorescence of the internalized cation probes was excited by scanning the 488 nm band of an Argon-ion laser through the computer-controlled galvanometric mirrors of a scanning laser confocal microscope (BioRad MRC-500). Emitted fluorescence was collected by a photomultiplier tube enclosed in the same housing and displayed as a 768 $\times$ 512 pixel resolution image through the framestore of the host computer (standard PC-AT). Optical section thickness was controlled by varying the size of an adjustable pinhole in the detector light-path. As whole-cell responses were the primary interest of the study optical sections between 5 and 10 μ m were generally viewed. To investigate gravitropism coleoptile tips were placed horizontally in the same chamber with the lateral aperture closed (see inset). The recording and analysis system remained unchanged. Coleoptiles were only scanned during the acquisition of images to avoid photobleaching of cellular fluo-3 or BCECF by laser illumination.

METHODS. Maize (*Zea mays* L. cv lochief) seeds were soaked in tap water (10 h) and then grown in vermiculite for 5 days at 26 °C in the dark. Coleoptiles were cut 1–2 cm below the apex and the primary leaf removed. The tips of coleoptiles were loaded with the esterified form of the Ca^{2+} indicator fluo-3 (1–4 μ M final concentration) for 1 h at room temperature (20–24 °C) in the dark. The removal of the outer waxy cuticle with adhesive tape was found to enhance this process and to be essential for loading and viewing of epidermal cells in experiments where coleoptiles were laid horizontally (gravitropism). Loading was terminated by rinsing in fresh tap water. The upper fluorescence limits were determined after the addition of the ionophore, A23187 (10 μ M final concentration), the lower limits were



established after adding EGTA (1 mM final concentration). Cellular fluorescence intensity was converted to an absolute change in dye saturation and free Ca^{2+} calculated from $[Ca^{2+}] = K_d [Ca \text{ fluo-3}] / [fluo-3]$ where the K_d in this experimental system was 437 nM and the absolute fluo-3 fluorescence enhancement was 36-fold (for further details see ref. 14). Internalized dye, isolated by mechanical homogenization, was found to be fully Ca^{2+} sensitive¹⁹. The heterogeneity of fluorescence intensities between cells is caused by the fact that organelles (for example, the vacuole) from which the dye is excluded are sometimes in the focal plane. Changes in $[Ca^{2+}]_{cyt}$ and pH_{cyt} can only be calculated from cells with the cytosol fully in the focal plane.

FIG. 2 Changes in fluo-3 fluorescence in the shaded segment of a unilaterally illuminated coleoptile tip. *a*, Before illumination (0 min), *b*, *c*, *d*, after 5, 10 and 15 min illumination respectively. Images were frame-averaged (16 frames). The outer most intense layer (red) represents the autofluorescence contribution of the waxy cuticle which remained constant. The colour bar represents a range of Ca^{2+} concentrations from 10 nM (dark blue) to 40 μM (pink).

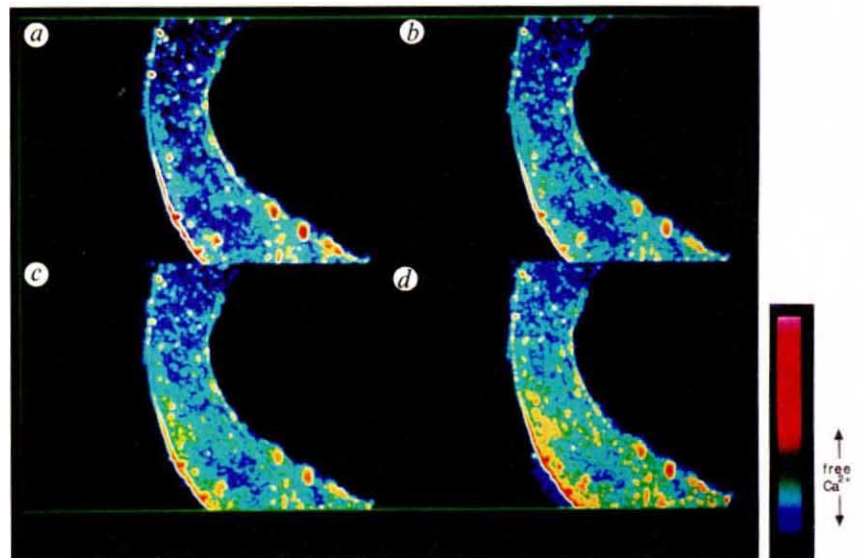


FIG. 3 Changes in fluo-3 fluorescence following gravitropic stimulus. The coleoptile was laid horizontally (*a*, time 0 min) and averaged images (16 frames) were captured 5 mm from the coleoptile apex 3 (*b*), 6 (*c*), and 9 min (*d*) after stimulation. The colour bar represents a range of Ca^{2+} concentrations from 10 nM (dark blue) to 40 μM (pink).

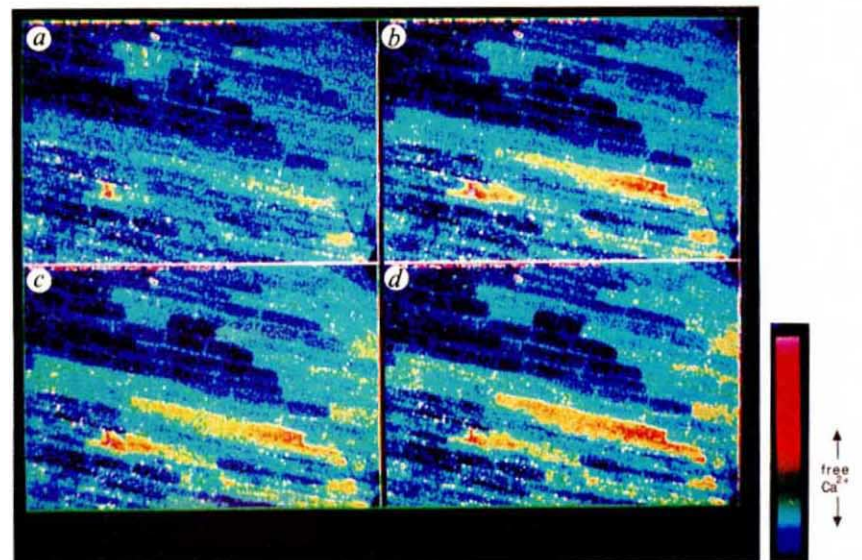
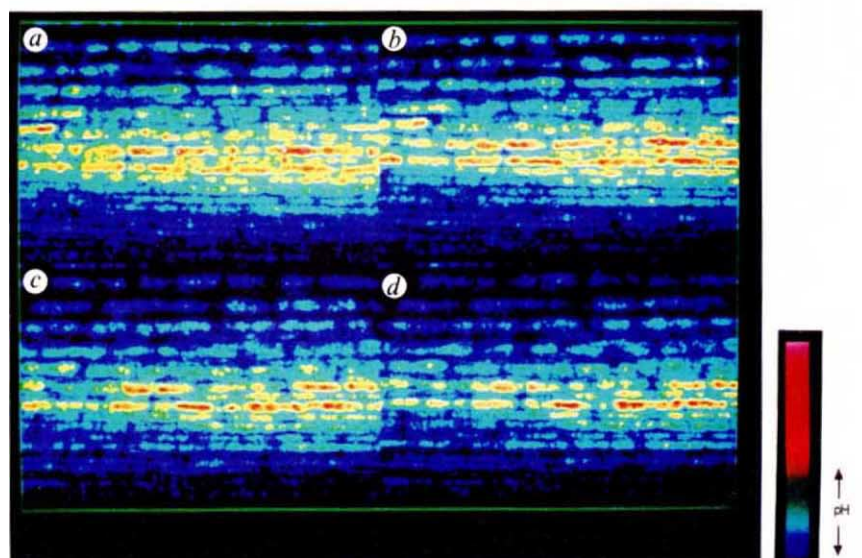


FIG. 4 Changes in BCECF fluorescence following gravitropic stimulus. The coleoptile was laid horizontally (*a*, time 0 min) and averaged images (16 frames) were captured 5 mm from the coleoptile apex after 3 (*b*), 6 (*c*), and 9 min (*d*). The colour bar represents a pH range from 7.0 (dark blue) to 7.8 (pink).

METHODS. Coleoptile tips were loaded with BCECF (1–2 μM final concentration) for 30 min in the dark at 20–24° followed by a rinse in fresh tap water. As a single wavelength mode was used, the BCECF fluorescence could not be directly measured in the *in vivo* system. *In vivo* dye responses were ascertained by lowering pH_{cyt} (for example, with fusicoccin¹²) and raising pH_{cyt} (for example, with procaine¹¹). Changes in pH_{cyt} were calculated from *in vitro* data, a linear relationship existing between fluorescence intensities and pH over a pH range of 6.4–7.6 (ref. 20).



cortical cells within 5 min and were only seen when the apical portion of the tip was illuminated. (It is the tip that senses the light; if the tip is removed or covered by an opaque cap the coleoptile will not bend².) The fluorescence intensity of tips loaded with fluo-3 and maintained in the dark over the same time period remained constant, indicating that the observed changes in distribution and intensity of Ca^{2+} -dependent fluorescence depended directly on unilateral illumination. The calculated $[\text{Ca}^{2+}]_{\text{cyt}}$ resting levels reported here are higher than those measured by microelectrodes in epidermal cells ($100 \mu\text{M}$)¹³. Our ionophoretic results represent a more heterogeneous cell population, including both epidermal and cortical cells. As well as varying from tissue to tissue, the resting $[\text{Ca}^{2+}]_{\text{cyt}}$ level will depend on growing conditions and Ca^{2+} supply.

Applying the same illumination protocol to BCECF-loaded cells, changes in pH_{cyt} from a resting level could be monitored (see Fig. 4). pH_{cyt} consistently dropped on the shaded side of the coleoptile tip during the viewing period (results not shown).

When coleoptile tips loaded with fluo-3 were laid horizontally within a darkened chamber, thus receiving a directional gravitational stimulus (see Fig. 1, inset), there were fluorescence changes in cells on the lower side. A rapid increase (within 3 min) in $[\text{Ca}^{2+}]_{\text{cyt}}$ from 255 nM to 370 nM occurred (Fig. 3a–b). The rise in Ca^{2+} remained constant after the initial increase although it varied with time from cell to cell (Fig. 3a–d).

In contrast pH_{cyt} continued to fall throughout the imaging period (Fig. 4a–d). Using the method described, the calculated change amounted to approximately 0.2 pH units. The technique did not enable us to observe cells on the upper side of the horizontal coleoptile but the pH_{cyt} and $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in the lower cells are consistent with changes observed during cell elongation. When coleoptiles were rotated through 180° (the former upper side becoming the lower side), similar changes in pH_{cyt} and $[\text{Ca}^{2+}]_{\text{cyt}}$ were again detected in the cells on the lower side.

The changes detected are consistent with a role for auxin in geo- and phototropism. Previous experiments with intracellular microelectrodes have shown that adding the auxin, indole-3-acetic acid (IAA), to maize coleoptiles decreases pH_{cyt} and increases $[\text{Ca}^{2+}]_{\text{cyt}}$ in epidermal cells^{11–13}. Using scanning laser confocal microscopy we have been able to see these changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ and pH_{cyt} in both epidermal and cortical cells after the addition of IAA (manuscript in preparation).

The changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ and pH_{cyt} during exposure to unilateral illumination and gravity reflect either altered sensitivity to auxin in specific regions of the coleoptile¹⁵, or alternatively, a redistribution of endogenous auxin (the Cholodny–Went hypothesis¹). The latter possibility would require the transport of auxin from illuminated cells to the shaded side or from the upper side to the lower side of a horizontal coleoptile. But as there was no apparent decrease in cation concentration (Ca^{2+} or H^+) in cells from which auxin would be removed following redistribution (unless the Ca^{2+} level had previously been raised above resting levels), a threshold for auxin effects on Ca^{2+} mobilization may exist, below which Ca^{2+} regulation is independent of absolute auxin concentration.

The distribution of auxin-regulated RNAs has been found to change when vertically orientated soybean hypocotyls are placed horizontally¹⁶. Within 20 min RNAs decrease on the upper side and increase on the lower side of the hypocotyls. The presence of the RNAs is highly correlated with cell elongation and they are expressed in both the epidermal and cortical layers. Our results suggest that increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ would precede these changes and that Ca^{2+} may therefore be a secondary messenger involved in the accumulation of such auxin-regulated RNAs. Such a mechanism may also involve the rapid breakdown of polyphosphoinositides for the mobilization of intracellular Ca^{2+} (ref. 17) and calmodulin-activated or Ca^{2+} -activated protein kinases^{10,18}. □

Received 8 February; accepted 16 March 1990.

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ACKNOWLEDGEMENTS. We thank P. J. Harris and H. R. Irving for discussion and for reviewing the manuscript, T. O. Morgan for the provision of experimental facilities at The University of Melbourne and T. Phillips, La Trobe University, for expert photographic assistance.

Evidence for the formation of heteromultimeric potassium channels in *Xenopus* oocytes

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POTASSIUM channels show a wide range of functional diversity^{1–3}. Nerve cells typically express a number of K^+ channels that differ in their kinetics, single-channel conductance, pharmacology, and sensitivity to voltage and second messengers. The cloning of the *Shaker* gene in *Drosophila*^{4–7}, and of related genes^{8–14}, has revealed that the encoded K^+ channel polypeptides resemble one of the four internally homologous domains of the α -subunits of Na^+ channels and Ca^{2+} channels^{15–18}, indicating that K^+ channels may form by the co-assembly of several polypeptides. In this report we provide evidence that the *Shaker* A-type K^+ channels expressed in *Xenopus* oocytes contain several *Shaker* polypeptides, that heteromultimeric channels may form through assembly of different channel polypeptides, that the kinetics or pharmacology of some heteromultimeric channels differ from those of homomultimeric channels, and that channel polypeptides from the fruit fly can co-assemble with homologous polypeptides from the rat. We suggest that heteromultimer formation may increase K^+ channel diversity beyond even the level expected from the large number of K^+ channel genes and alternative splicing products^{4,5,19–25}.

To test whether K^+ channels are composed of multimers we selected wild-type and mutant products of *Shaker* which, individually, produce channels with easily distinguishable kinetic behavior. We co-injected their messenger RNAs into *Xenopus* oocytes to investigate whether the expressed currents could be accounted for by the independent expression of the two products. The first *Shaker* variants that we combined were ShA and a mutant of ShB, ShB($\Delta 17$ –25), from which amino-acids 17–25 have been deleted. As reported previously, ShA currents inactivate rapidly and recover slowly from inactivation^{22,23} (Figs 1 and 3, Table 1). In contrast ShB($\Delta 17$ –25) currents, similar to currents due to other deletion mutations within the amino-terminal region²⁶, inactivated about one hundred times more slowly (Fig. 1, Table 1). Oocytes co-injected with ShA mRNA and ShB($\Delta 17$ –25) mRNA expressed currents that inactivated much more slowly than ShA currents and much more rapidly than ShB($\Delta 17$ –25) currents (Fig. 1a, Table 1) and

that could not be approximated by the algebraic sum of the two (Fig. 1a). This indicates that currents in the co-injected oocytes cannot be accounted for by the independent expression of ShA channels and ShB($\Delta 17-25$) channels. Similarly, recovery from inactivation in co-injected oocytes was much faster than that for ShA and much slower than that for ShB($\Delta 17-25$) (Fig. 1b). As shown in Fig. 1c, currents from co-injected oocytes recovered much more rapidly at -80 mV than ShA currents. As these currents also inactivated rapidly, unlike ShB($\Delta 17-25$) currents, they could not be due to either ShA or ShB($\Delta 17-25$) channels. Therefore, the novel kinetics of onset of inactivation and recovery from inactivation in co-injected oocytes indicate that co-assembly of the two variant polypeptides occurs, leading to the formation of channels that have distinct kinetics.

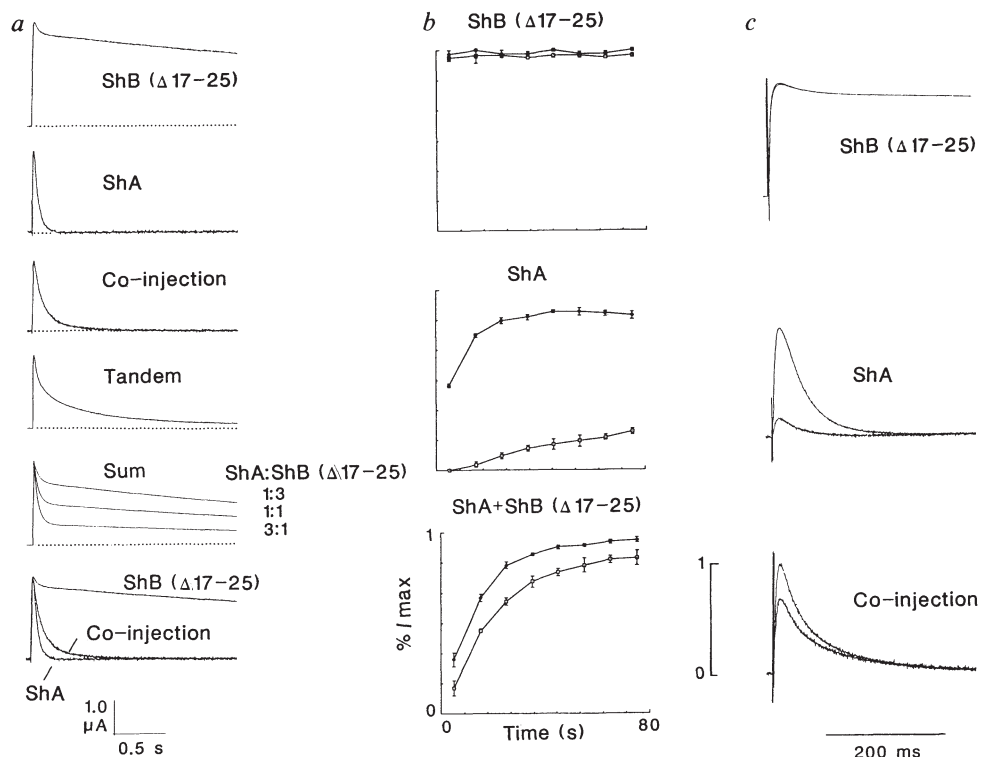
As a direct test for the formation of heteromultimeric channels, single-channel recording was done. Like the A-type channels from *Drosophila* myocytes^{27,28}, ShA channels produced short bursts of activity once or twice early in the 180 ms depolarizing pulse (Fig. 2a). In contrast, ShB($\Delta 17-25$) channels produced long bursts of activity which occurred throughout the pulse (Fig. 2b). This difference in the duration and the probabil-

ity of recurrence of the bursts explains the slower inactivation rates for ShB($\Delta 17-25$) channels, as seen in the ensemble average (Fig. 2b and f). Differences in temperature between the patch recordings (22°C) and the two-electrode voltage clamp recordings (11°C) probably account for the generally faster inactivation kinetics of the patch recordings. Channels expressed in co-injected oocytes were intermediate between those of ShA channels and ShB($\Delta 17-25$) channels with respect to both the probability of recurrence of bursts and the recovery from inactivation (Fig. 2c and d and legend to Fig. 2). These studies have therefore revealed the presence of channel molecules with novel properties in the co-injected oocytes. As ensemble-averages of membrane patches that contained either one active channel, or 5–20 active channels from the co-injected oocytes, possessed kinetic properties intermediate between those of ShA and ShB($\Delta 17-25$) channels, it is likely that the novel kinetic components observed in currents from co-injected oocytes are mostly due to the distinct kinetic behaviour of single channels.

If K^+ channel assembly occurs by the independent assortment of several subunits (possibly four, by analogy with Na^+ and Ca^{2+} channels), then co-expression of two *Shaker* variants may

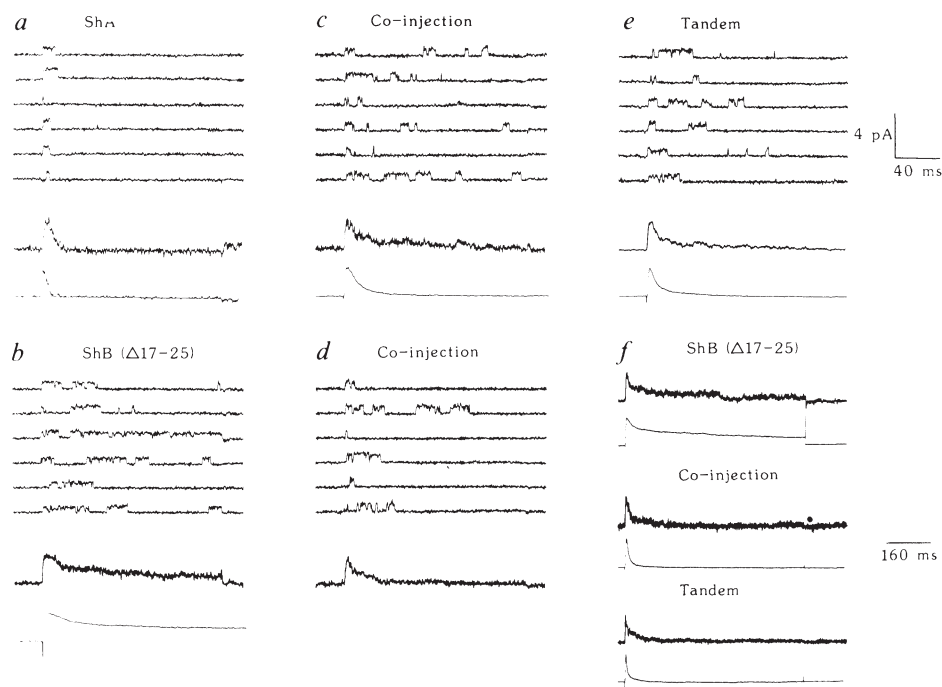
FIG. 1 Co-expression of ShA and the deletion mutant ShB($\Delta 17-25$) results in whole-cell currents with novel kinetics. (a) Currents evoked by 2 s pulses of depolarization from -100 mV to $+40$ mV, delivered once every 80 s, in oocytes injected with 10 ng of mRNA of either ShB($\Delta 17-25$) alone or ShA alone, with a combination of 5 ng of each (co-injection), or with 10 ng of mRNA from the tandem construct ShA-ShB($\Delta 17-25$). In the bottom panel, currents from co-injected oocytes and from oocytes injected with ShA or ShB($\Delta 17-25$) are normalized and superimposed to illustrate the differences in their wave forms. Also shown are algebraic sums of ShA and ShB($\Delta 17-25$) currents (using mean values from Table 1) in ratios of 1:3, 1:1 and 3:1. The lack of correspondence between the algebraic sums and the observed currents in co-injected oocytes indicates that these currents can not be accounted for by independent expression of the two channel types and that, instead, they are probably due to heteromultimeric channel formation. (b) The rate of recovery from inactivation for currents in oocytes injected with ShB($\Delta 17-25$) mRNA alone ($N=5$), ShA mRNA alone ($N=7$) or a co-injection of a 1:1 mixture of the two ($N=7$). After the initial pulse of depolarization to $+40$ mV for 0.4 s, membrane potential was held at either -80 mV (open symbols) or -100 mV (filled symbols) for variable duration before a second pulse to $+40$ mV was given. Peak current amplitude of the second pulse is normalized to that of the initial pulse and plotted against the interval between these pulses. (c) Current evoked by a second pulse following 80 s of recovery at -80 mV is normalized to and superimposed with the current evoked by the initial pulse at time 0. Note that rapidly inactivating currents in the co-injected oocytes recover much more quickly than those in ShA injected oocytes.

METHODS. Transcription, injection and two-electrode voltage-clamping as described previously^{22,23}, except that one fifth the concentration of unmethylated CAP analogue was used. Sampling and analysis were performed on an AST 386C using PCLAMP and an Axon Instruments TL-1 A/D converter. Exponential fits used PCLAMP's least-squares minimization procedure with adequacy of the fit judged by eye. The solutions used in the patch recordings were as in ref. 28. Mutations and tandem constructs were confirmed in DNA sequencing, except the tandem ShB-ShB, which was verified by cell-free



translation to make a single protein product of twice the molecular weight of the ShB protein. Batches of oocytes in which any endogenous slow outward currents were evident at $+40$ mV and oocytes having membrane resistances of less than 0.5 Mohms were excluded from the data set. Only currents of 1–3 μA were used for the kinetic measurements. Series resistance, measured as 500 ohms (L. Timpe, unpublished observation), was not compensated for, resulting in a maximal predicted error of 1.5 mV for the largest currents measured. Records were leak-subtracted using scaled 20 mV hyperpolarizing templates. All the two-electrode voltage-clamp experiments (Figs 1, 3 and 4) were done at $11 \pm 1^\circ\text{C}$. The patch recordings (Fig. 2) were done at $22 \pm 2^\circ\text{C}$ using a List EPC7, and analyzed on an Indec PDP/1173. The recordings were done in the outside-out configuration as in ref. 28. Leak subtraction was done using averages of steps during which there were no channel openings. Both the two-electrode voltage-clamp recordings and the patch recordings were sampled at 5 kHz and filtered at 1 kHz. All values are expressed as mean $\pm$ standard-error of the mean, except where indicated.

FIG. 2 Patch recordings from oocytes injected with 2 ng of mRNA of either ShA alone or ShB($\Delta 17-25$) alone, with 1 ng of each (co-injection), or with 2 ng of mRNA from the tandem construct ShA-ShB($\Delta 17-25$). In panels *a-e* currents from patches containing a single channel are displayed immediately above the ensemble-average from that patch. Depolarizations were from -120 mV to $+40$ mV for 180 ms. The bottom traces in *a*, *b*, *c*, and *e* are averages from patches that contain $\sim 5-20$ channels. In *f*, single channel ensemble averages for the same channels as shown in *b*, *c* and *e* but evoked by longer (720 ms) steps are displayed above average currents from multi-channel patches. Panels *c* and *d* represent two different patches from co-injected oocytes. Averages of single and multi-channel activity have been normalized for comparison. The general agreement between single and multi-channel averages indicates that the kinetic differences between currents in co-injected oocytes and those injected with one mRNA variant alone are largely due to different kinetic properties of individual channel molecules. The intermediate behaviour was most obvious in terms of the probability of the channel being in the bursting state (total burst duration) in the last 130 ms of the pulse (P_{50-180}). Bursts were defined as openings interrupted by less than 1 ms closed times, as in ref. 27. Whereas ShA channels had a P_{50-180} of $1.2 \pm 1.5\%$ ($N=9$) and ShB($\Delta 17-25$) had a P_{50-180} of $33.5 \pm 6.5\%$ ($N=9$), channels in co-injected oocytes had a P_{50-180} of $13.2 \pm 9.2\%$ ($N=10$) (values



are mean $\pm$ standard deviation). Channels from co-injected oocytes also differed from ShA channels in their recovery from inactivation, in that they could be activated repeatedly if the membrane potential was held at -100 mV for 4 s between pulses of depolarization. The ShA channels required at least 10 s of hyperpolarization at -120 mV to recover from inactivation.

result in many combinations with different ratios of the two subunit types (for example, 4:0, 3:1, 2:2, 1:3, 0:4, for a four subunit channel). To constrain the stoichiometry of the heteromultimers and express a uniform population of mixed subunit channels we constructed fusion genes linking one copy of each of the two *Shaker* variants in a single open reading frame. In these tandem dimers a linker of ten glutamine residues connects the last residue of the first *Shaker* polypeptide with the sixth residue of the second *Shaker* polypeptide. Tandem constructs of ShA and ShB($\Delta 17-25$) induced currents that were qualitatively similar to currents in the co-injected oocytes (Fig. 1a). Moreover, channels composed of these tandem constructs

had single-channel conductances and kinetic properties very similar to those observed in co-injected oocytes (Fig. 2). As a control, currents generated by tandem homodimers of ShB were examined and found to resemble closely the currents from ShB monomers (Fig. 3a, Table 1). Although we could not be certain that the channels from which we recorded were composed of intact tandem dimers of *Shaker* polypeptides, these findings are consistent with the expectation that mRNA of heteromeric tandem dimers induces the formation of heteromultimeric channels in which both portions of the dimeric construct participate in channel formation. If this is the case, these results indicate that K^+ channels can be composed of an even number of *Shaker*

TABLE 1 Macroscopic inactivation kinetics

mRNA	A_1	τ_1	A_2	τ_2	A_3	τ_3	N
ShA	1.00	40.9 ± 1.8	—	—	—	—	12
ShB	0.76 ± 0.02	15.9 ± 0.7	0.24 ± 0.02	$1,323 \pm 97$	—	—	9
Tandem ShB-ShB	0.66 ± 0.02	18.9 ± 1.0	0.34 ± 0.02	$1,909 \pm 17$	—	—	3
ShB($\Delta 17-25$)	0.07 ± 0.12	40.5 ± 13.9	0.93 ± 0.12	$4,482 \pm 531$	—	—	8
Co-injection ShB + ShA	0.47 ± 0.02	26.6 ± 3.0	0.43 ± 0.02	88 ± 7	0.10 ± 0.02	370 ± 65	5
Tandem ShB-ShA	0.30 ± 0.02	23.8 ± 1.4	0.49 ± 0.01	132 ± 6	0.21 ± 0.03	444 ± 21	6
Co-injection ShA + ShB($\Delta 17-25$)	0.45 ± 0.03	41.2 ± 2.9	0.44 ± 0.02	122 ± 6	0.11 ± 0.02	481 ± 26	6
Tandem ShA-ShB($\Delta 17-25$)	0.27 ± 0.02	24.6 ± 0.6	0.25 ± 0.04	161 ± 4	0.48 ± 0.05	811 ± 50	3

Values measured from experimental fits to the onset of inactivation, in oocytes injected with different mRNAs. Currents evoked by 0.4 s depolarizing pulses (from -100 mV to $+40$ mV) were used to measure time-constants of the faster-inactivating components, whereas currents evoked by 2 s and 10 s pulses were used to measure the time-constants of slower-inactivating components. The combined results were then tested on currents evoked by pulses of all three durations to ensure that the composite fit described the overall kinetics properly. Only currents of $1-3 \mu A$ were used (Fig. 1 legend). The minimum number of exponentials required for an adequate fit of the currents ranges from one to three. The time-constants are τ_1, τ_2, τ_3 (in ms) and A_1, A_2, A_3 represent the relative contribution of each component to the peak, so that the current as a function of time (t) (with the peak amplitude normalized to 1.0) matches the curve of $A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + A_3 \exp(-t/\tau_3)$. The means and standard errors of the mean are shown. N , total number of oocytes tested.

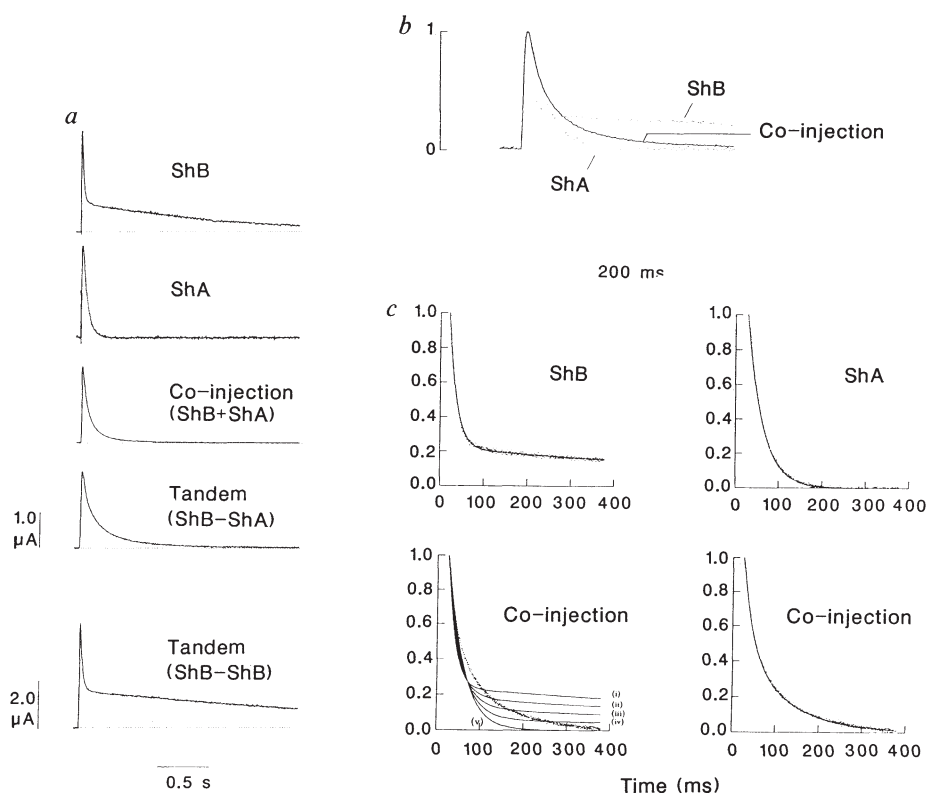
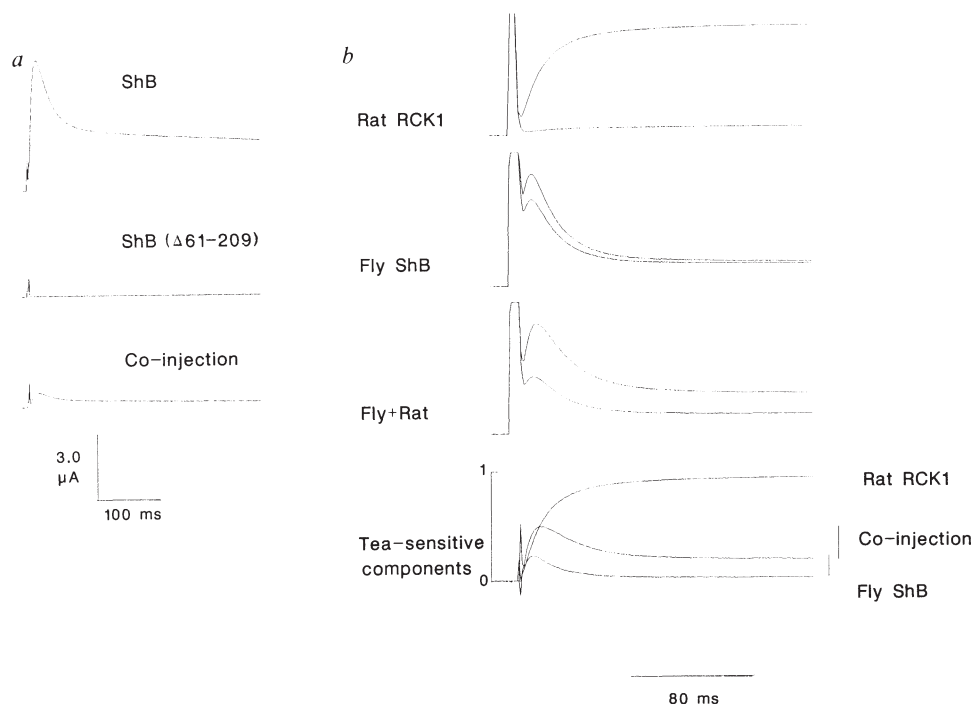


FIG. 3 Distinct kinetics of currents in oocytes co-expressing two wild-type *Shaker* products. (a) currents evoked by 2 s depolarizations from -100 to $+40$ mV in oocytes injected with 10 ng total mRNA from ShB alone, ShA alone, a 1:1 mixture of the two and mRNA from a ShB-ShA tandem dimer and a ShB-ShB tandem dimer. (b) superposition of normalized traces of ShA, ShB and the co-injection show that about half of the co-injection current amplitude is due to components with inactivation rates that differ from those of both ShA and ShB currents. (c) One or two exponential fits to currents evoked by 4 s depolarizations from -100 to $+40$ mV for ShB, ShA and the 1:1 co-injection of ShA and ShB. ShA and ShB currents were adequately fitted by one and two exponentials, respectively (Table 1). The co-injection currents differed from the ShB current (i) or the ShA current (v), or the sum of ShB and ShA currents in a 3:1 (ii), 1:1 (iii) or 1:3 (iv) ratio. (Lower left panel). Much better fits for these currents were obtained using three exponentials with distinct amplitudes and time-constants (lower right panel, see Table 1). The inadequacy of the algebraic sum of ShA and ShB currents in describing currents in the co-injected oocytes indicates that currents in the co-injected oocytes can not be due only to independent expression of ShA channels and ShB channels.

FIG. 4 a, Association between wild-type and mutant *Shaker* products. Injection of mRNA of either ShB alone (5 ng) or of mRNA of the deletion mutant ShB($\Delta 61-209$) alone (10 ng), or co-injection of ShB (5 ng) and ShB($\Delta 61-209$) (10 ng) mRNAs into *Xenopus* oocytes produced peak currents of 4.2 ± 0.6 μ A ($N=14$), 0 μ A ($N=6$) and 0.5 ± 0.1 μ A ($N=9$), respectively. Membrane potential was stepped from -100 mV to $+40$ mV for 0.4 s, once every 80 s. b, Hybrid channels formed with fruit fly and rat channel polypeptides. Comparison of sensitivity to TEA of currents from oocytes injected with RCK1 mRNA alone (20 ng), ShB mRNA alone (10 ng), or half the amount of each, combined. These amounts of mRNA evoked peak currents of 2.6 ± 0.5 μ A ($N=7$), 2.4 ± 0.3 μ A ($N=8$) and 2.5 ± 0.5 μ A ($N=7$) for RCK1, ShB and the co-injection, respectively. All currents shown in Fig. 4b are normalized, so that the peak currents before introduction of 10 mM TEA have the same amplitude. The top three panels are superimposed averages of currents (from RCK1, ShB, or co-injection) evoked by 0.2 s depolarizations from -100 to $+40$ mV, before and after switching to superfusion with MBSH (in which 10 mM of the NaCl was substituted with 10 mM TEA-Cl). The bottom panel shows superposition of the TEA-sensitive component (that is, the current in the presence of TEA subtracted from the control current) from these three oocytes. Bars on the right indicate the fraction of these currents that are rapidly inactivating and TEA-sensitive. Note that the fast-inactivating com-



ponent in the co-injected oocytes cannot be due to RCK1, which does not show fast inactivation, nor can it be due to ShB as it is twice as sensitive to 10 mM TEA as ShB. Values in the text represent per cent inhibition either of current at the end of the depolarizing step (for RCK1) or of the fast component of inactivation (for ShB and the co-injection) measured by fitting exponentials as in Table 1.

polypeptides that have both termini on the same side of the membrane, similar to the arrangement of the Na⁺ channel α -subunit^{16,17}. Definitive characterizations of the subunit structure of these K⁺ channels and the folding of the channel polypeptides must be carried out through biochemical and structural analyses.

Having obtained evidence that the combination of a wild-type and a mutant *Shaker* product gives rise to heteromultimeric channels with distinct kinetic properties, we extended the study to determine whether two wild-type *Shaker* products can also form heteromultimeric channels. To address this question, we injected oocytes with mRNA of ShA and ShB (that differ only at their carboxy termini, due to alternative splicing^{4,5,15}) and compared the macroscopic inactivation rates quantitatively. We also examined currents induced by tandem constructs of ShA and ShB. Currents measured in co-injected oocytes or in oocytes injected with the tandem construct decayed with time-constants different from those of ShA or ShB currents and could not be accounted for by the algebraic sum of ShA and ShB currents (Fig. 3, Table 1), indicating that heteromultimeric channels were indeed formed through co-assembly of ShA and ShB polypeptides. Whether similar heteromultimeric channels also form *in vivo* can only be determined by analysing the expression patterns of the different *Shaker* products and by detailed comparative electrophysiological studies of *Drosophila* neurons and muscles that express *Shaker* A-type channels²⁷⁻³¹. Nonetheless, the evidence for heteromultimeric channel formation in oocytes strongly suggests that this A-type K⁺ channel contains several *Shaker* products or other related polypeptides.

The multimeric nature of A-type K⁺ channels could explain several observations derived from genetic studies of the *Shaker* locus, including the prevalence of dominant mutations³¹⁻³³ and interactions between specific *Shaker* alleles³⁴. In fact, combining a wild-type *Shaker* product, ShB, with mRNA from a null mutant (such as ShB(Δ 61-209) in which amino acids 61-209 are deleted) that by itself induced no K⁺ currents in oocytes, caused a great reduction of the ShB currents (Fig. 4a). Because injecting two or four times as much ShB mRNA roughly doubled or quadrupled the K⁺ current amplitude (data not shown), the reduction of ShB current in co-injected oocytes is not likely to result from competition between ShB and ShB(Δ 61-209) mRNA for the translation machinery. It is more likely that the null mutant interferes with the production of functional K⁺ channels by associating with the wild-type ShB polypeptides, though competition between mutant and wild-type *Shaker* proteins at the level of membrane insertion remains a possibility.

Having obtained evidence for the co-assembly of two *Shaker* polypeptides that differ only in their amino and carboxy termini,

we next determined whether more distantly related polypeptides could also co-assemble and form functional channels. The ShB mRNA was therefore injected into the same oocytes as mRNA from a homologue isolated from the rat brain⁹⁻¹², RCK1, that has about 65% amino-acid identity with ShB. The RCK1 currents did not inactivate rapidly and were almost completely inhibited ($93.2 \pm 1.1\%$, $N = 8$) by 10 mM tetraethylammonium (TEA), as reported previously^{10,12}. By contrast, ShB currents inactivated rapidly²²⁻²⁴ and were inhibited to a much lesser extent ($25.4 \pm 1.4\%$, $N = 8$) by 10 mM TEA. Unlike either RCK1 or ShB currents, currents in co-injected oocytes contained a rapidly inactivating component that was half-inhibited ($49.0 \pm 2.9\%$, $N = 5$) in 10 mM TEA (Fig. 4b). The rapidly inactivating channels with intermediate sensitivity to TEA are probably composed of both fruit fly and rat channel polypeptides.

In summary, we present evidence here indicating that the *Shaker* A-type K⁺ channel formed in *Xenopus* oocytes is a multimeric protein, probably composed of an even number of similar or identical subunits. Moreover, assembly of different subunits can produce channels with distinct kinetics and/or pharmacology. We do not know whether heteromultimeric channels also form *in vivo*, though it is likely and may partially explain the observed heterogeneity of K⁺ channels.

Although RCK1 and ShB(Δ 17-25) induce currents that do not inactivate rapidly, co-expression of either with a wild-type *Shaker* product gives rise to rapidly inactivating channels. Thus, the ability to undergo fast inactivation appears to be dominant in these heteromultimeric channels. It is important to note that properties of channels formed in *Xenopus* oocytes do not necessarily reflect channel properties *in vivo*, where the appearance of various transcripts may be timed to permit or restrict co-assembly. For example, although several rat brain K⁺ channel mRNA species are each found to induce slow-inactivating currents in *Xenopus* oocytes⁹⁻¹³, it is possible that the polypeptides that they encode combine with other channel components *in vivo* and result in rapidly inactivating currents. If heteromultimeric channels do form naturally, the control of channel stoichiometry might represent another intriguing form of channel regulation.

The functional association of channel components from the fruit fly and the rat indicate that, even though these species diverged 600 million years ago, these channel components are similar enough in their tertiary structures to allow functional subunit interactions. Grouping those polypeptides that can assemble with one another and form channels might be one way of applying structural criteria in the classification of these K⁺ channel components. □

Received 19 February; accepted 17 April 1990.

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ACKNOWLEDGEMENTS. We are grateful to Dr Jeffrey Lansman, Paul Slesinger, and Alfredo Franco for use of their patch-clamp recording apparatus and for advice and help, to Dr David McKinnon for providing the RCK1 clone, to Katherine Bornschlegel, Sharon Fried, Denise Muhrad, and Rabiya Tuma for technical assistance, Drs Richard Aldrich, Thomas Schwarz, Leslie Timpe, Edward Giniger, and other colleagues for comments on the manuscript, Drs Ethan Bier and Rolf Bodmer for helpful discussions, Katherine Prewitt for preparing the manuscript and Larry Ackerman for the photography. E.Y.J. is an MDA postdoctoral fellow. Y.N.J. and L.Y.J. are Howard Hughes Investigators. This work is supported by the NIH.

Heteromultimeric channels formed by rat brain potassium-channel proteins

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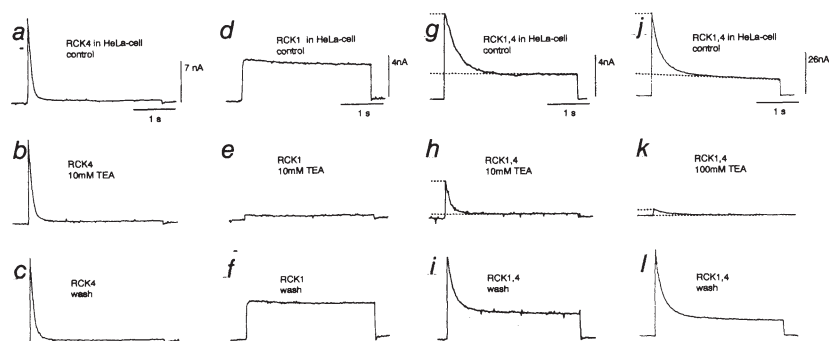
AN important step towards understanding the molecular basis of the functional diversity of voltage-gated K⁺ channels in the mammalian brain has been the discovery of a family of genes encoding rat brain K⁺ channel-forming (RCK) proteins^{1–6}. All species of these RCK proteins form homomultimeric voltage-gated K⁺ channels with distinct functional characteristics in *Xenopus laevis* oocytes following injection of the respective cRNAs². RCK-specific mRNAs are coexpressed in several regions of the brain⁷, suggesting that RCK proteins also assemble into heteromultimeric K⁺ channels. In addition expression experiments with fractionated poly(A)⁺ mRNA have suggested that heteromultimeric K⁺ channels may occur in mammalian brain⁸. We report here that heteromultimeric K⁺ channels composed of two different RCK proteins (RCK1 and RCK4) assemble after cotransfection of HeLa cells with the corresponding cDNAs and after coinjection of the corresponding cRNAs into *Xenopus* oocytes. The heteromultimeric RCK1, 4 channel mediates a transient potassium outward current, similar to the RCK4 channel but inactivates more slowly, has a

larger conductance and is more sensitive to block by dendrotoxin and tetraethylammonium chloride.

Transfection of HeLa cells with plasmids containing cDNAs encoding RCK1 (pMSVR1) or RCK4 (pMSVR4) produced channels with properties similar to those observed in oocytes², though untransfected HeLa cells had no outwardly rectifying K⁺ channels. Currents through RCK4 channels were transient and completely insensitive to tetraethylammonium chloride (TEA) (Fig. 1a–c) whereas RCK1 channels mediated a non-inactivating current highly sensitive to TEA (dissociation constant (K_D) = 0.6 mM, see ref. 2; Fig. 1d–f). Figure 1g shows a record of an outward K⁺ current measured in a HeLa cell co-transfected with RCK1 cDNA and RCK4 cDNA. A depolarizing voltage step to 0 mV evoked an outward current consisting of an initial transient current followed by a non-inactivating component. Because the non-inactivating component was reversibly blocked by 10 mM extracellular TEA (Fig. 1h, i) similar to that in HeLa cells expressing RCK1 channels (Fig. 1d–f), it is likely that this component is mediated either by homomultimeric RCK1 channels formed on cotransfection with both cDNAs or by heteromultimeric channels indistinguishable from RCK1 channels. In contrast to the non-inactivating component, the transient component has pharmacological differences from that seen in homomultimeric RCK4 channels. The transient component was half-blocked by 10 mM TEA (Fig. 1h, peak amplitude reduced to 50 ± 18% of control, $n = 3$) and reduced by 100 nM dendrotoxin (DTX) (peak amplitude reduced to 62 ± 8% of control, $n = 4$), whereas RCK4 channels were completely insensitive to those concentrations (for TEA see Fig. 1a–c). The transient component was almost completely blocked by 100 mM TEA (Fig. 1j–l) whereas RCK4 channels were unaffected². Similar pharmacological differences were observed in oocytes coinjected with RCK1- and RCK4-specific cRNAs and with RCK4-specific cRNA, respectively.

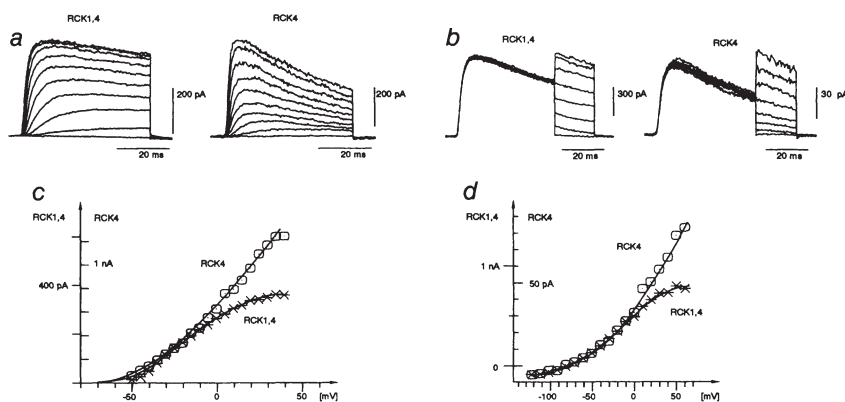
FIG. 1 Action of TEA on K⁺ currents in HeLa cells transfected with plasmids carrying RCK cDNA. **a**, Transfection with plasmid carrying RCK4 (pMSVR4) cDNA. Transient current in response to a 3.2 s step from –80 mV membrane potential to 0 mV. **b**, Response of the same cell to the same pulse protocol as in (a) following bath application of 10 mM TEA. No effect on the current. **c**, Same cell, same pulse protocol as in (a) and (b) but after wash-out of TEA. **d**, Transfection with plasmid carrying RCK1 cDNA (pMSVR1). Non-inactivating current mediated by RCK1 channels, pulse protocol as in (a). **e**, Same cell and same pulse in the presence of 10 mM TEA. The current is almost completely blocked. **f**, Same cell as in (d) and (e) after wash-out of TEA. **g**, Cotransfection with plasmids harbouring RCK1 cDNA (pMSVR1) and RCK4 (pMSVR4) cDNA. **h**, Same cell and same pulse in the presence of 10 mM TEA; non-inactivating component almost completely blocked. The transient current calculated as the difference between the peak and the dotted line is reduced to 50.3 ± 18%, $n = 3$. **i**, Same cell, same pulse protocol as in (g) and (h) but after wash-out of TEA. **j**, Same cotransfection and same pulse as in (g) but different cell. **k**, Same cell as in (j) following bath application of 100 mM TEA; non-inactivating component completely blocked, transient current almost completely blocked. **l**, same cell as in (j) but after wash-out of TEA. Calibration for each column is shown in upper panels.

METHODS. The cDNA was obtained by subcloning an EcoRI fragment of RCK1 cDNA (nucleotides –256–3575) (ref. 3) into Bluescript (pRCK1). Plasmid pRCK1 was digested with *Sma*I/*Hind*III and filled in with Klenow fragment of DNA polymerase I. The isolated fragment (nucleotides –45–1649) (ref. 3) was inserted between the SV40 promoter region and the SV40 3' late region⁹ of pMSV (ref. 10) by blunt-end ligation. pMSV DNA was derived from pML2 DNA¹⁰. It contains the SV40 promoter region (a 342 base pair (bp) *Hind*III/*Pvu*II fragment) and the 3' region of SV40 late genes (a 273 bp *Bcl*I/*Bam*HI fragment)^{9–11}. RCK4 cDNA was subcloned into Bluescript (pRCK4) (ref. 2). Noncoding 5' and 3' regions were deleted by religation,



resulting in a plasmid containing RCK4 cDNA sequences nucleotides –85–2171 (pRCK4c). This DNA was digested with *Hind*III and *Bam*HI. The resulting *Hind*III/*Bam*HI RCK4c fragment was ligated with *Hind*III/*Bgl*II-cut pMSV DNA. The final constructs were checked by restriction enzyme digests. HeLa cells¹² cultured by a standard protocol¹² were transiently transfected according to the method of Chen and Okayama¹³. In untransfected HeLa cells the presence of detectable amounts of RCK1 RNA was excluded with northern blots. Current measurements in HeLa were made at 20 °C in the whole-cell recording configuration 24–48 h after transfection using standard patch clamp techniques¹⁴. The bathing solution contained (in mM): NaCl 135; KCl 5.4; CaCl₂ 1.8; MgCl₂ 1; HEPES buffer 5 (pH 7.2). The intracellular solution contained (in mM): KCl 130; CaCl₂ 2; MgCl₂ 2; EGTA 10; HEPES buffer 10 (pH 7.2). Currents were filtered at 120 Hz, sampled at 62.5 Hz and analysed on a VME-bus computer. All transient recordings were corrected for leakage and capacitive currents by the P/4 method¹⁵. Control experiments with native HeLa cells did not show voltage-activated K⁺ currents of the type seen in transfected cells.

FIG. 2 Activation of K^+ currents in *Xenopus laevis* oocytes injected with different RCK-specific cRNAs. Current measurements from cell-attached patches. **a**, Current responses to depolarizing test pulses of 50 ms in oocytes coinjected with cRNAs encoding RCK1 and RCK4 (left family of traces) or of oocytes injected with RCK4 specific cRNA (right family of traces). The holding potential was -80 mV, the test pulses ranged -50 – 40 mV in 10 mV intervals. The currents were filtered at 3 kHz (-3 dB) and digitized at 4 kHz. **b**, Tail-current experiments to obtain the instantaneous current-voltage relation (I - V) in response to voltage steps following an activating pulse to 0 mV. Voltage steps -80 – 60 mV in 20 mV increments. The I - V relation of the instantaneous current in coinjected oocytes is linear between -60 and 20 mV and saturates at potentials positive to 20 mV (left panel). In oocytes expressing RCK4 channels the instantaneous I - V relation is superlinear (right panel). **c**, Plot of the peak amplitudes of the transient currents versus voltage obtained from the measurements shown in (**a**). To facilitate comparison the amplitudes were scaled to yield the same slope at -25 mV. The corresponding current values are indicated at the ordinate. **d**, Plot of the peak amplitudes of the instantaneous currents versus voltage obtained from a measurement as shown in (**b**) but beginning at -120 mV with 10 mV increments. To facilitate comparison the amplitudes were scaled to yield the same slope at -80 mV.



The corresponding current values are indicated at the ordinate. **METHODS.** Capped run-off cRNA was synthesized by a standard protocol¹⁶. A mixture of equal parts of RCK1- and RCK4-specific cRNA was coinjected into *Xenopus* oocytes². After 2–4 days the oocytes expressed K^+ channels at a high density. Current measurements from cell-attached patches in oocytes were made as described², except that 10 mM TEA was added to the pipette solution.

These differences in DTX and TEA sensitivity suggest that the transient component is mediated by a novel, heteromultimeric K^+ channel composed of RCK1 and RCK4 subunits, rather than by homomultimeric RCK4 channels.

This view is further supported by differences in gating and conductance properties of the channels mediating the transient currents. The experiments were carried out in cell-attached macro-patches on oocytes where voltage control was better than in whole-cell recordings in HeLa cells or oocytes². Figure 2 shows transient currents recorded in the presence of TEA (10 mM) to block non-inactivating currents. In oocytes coinjected with RCK1- and RCK4-specific cRNA the peak of the transient current saturated at potentials positive to 20 mV and currents showed little inactivation during the test pulse (Fig. 2a, c). In contrast, in oocytes injected only with RCK4-specific cRNA the peak of the transient current showed no saturation up to 40 mV and began to inactivate during the test pulse (Fig. 2a, c). As the instantaneous current-voltage relationship also saturated for RCK1,4 channels (Fig. 2b, d), but not for RCK4 channels (Fig. 2b, d), the differences in saturation of the peak-transient currents results from different open channel characteristics and not from different activation kinetics. To evaluate whether these properties or the formation of the heteromultimeric channels depend on the expression system, we also compared whole-cell currents recorded in HeLa cells transfected with RCK4 and with RCK1 and RCK4 cRNA. The differences in saturation of the peak-transient currents were also found in the two types of transfected HeLa cells.

The steady-state inactivation behaviour, measured by responses to test pulses to 0 mV from different prepulse potentials (prepulse duration of 25 s) showed that the inactivation curves in coinjected oocytes were slightly steeper (change in prepulse potential for an e-fold reduction of the current: $a_h = 6.8 \pm 0.9$ mV) and were shifted by about 15 mV towards more positive potentials (half inactivation voltages $V_{1/2} = -58.3 \pm 2.0$ mV, $n = 3$) in comparison with currents mediated by RCK4 channels ($a_h = 12.8 \pm 2.2$ mV, $V_{1/2} = 73.6 \pm 5.2$ mV, $n = 6$). The inactivation time constants of the transient currents, measured at 0 mV in presence of 10 mM TEA, were different in both oocytes (228 ± 59 ms, $n = 5$, for coinjected cells and 109 ± 58 ms, $n = 8$, for RCK4 channels) and HeLa cells (155 ± 42 ms, $n = 5$ in cotransfected cells and 68 ± 19 ms, $n = 6$ for RCK4 channels). Figure 3 shows the time course of recovery from inactivation.

Transient currents in coinjected oocytes (Fig. 3a) recover more than two times faster from inactivation caused by a depolarizing pulse than those mediated by RCK4 channels (Fig. 3b).

Voltage-activated single channel currents (Fig. 4a) in coinjected oocytes mediate a rapidly inactivating current (Fig. 4b)

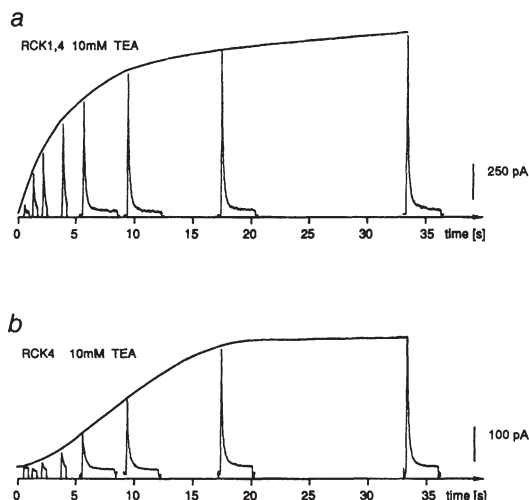


FIG. 3 Recovery of transient currents from inactivation. Recordings from cell-attached patches. **a**, Oocyte coinjected with RCK1- and RCK4-specific cRNAs. Following a pause of 180 s, a conditioning pulse (5 s) to 0 mV inactivated all channels in the membrane patch. The degree of recovery from inactivation as percentage of the current elicited by the conditioning pulse was measured with a test pulse to 0 mV at variable times. Paired conditioning and test pulses were made to avoid contributions of test-pulse-induced inactivation. The duration of test pulses was 0.5 or 3.2 s. The time to 50% recovery was 5.7 ± 1.8 s ($n = 3$). **b**, Oocyte injected with RCK4-specific cRNA. The time to 50% recovery was 16.5 ± 2.5 s ($n = 4$). Experimental conditions as described in Fig. 2. TEA (10 mM) was added to the filling solution of the recording pipette. Comparable differences in the recovery from inactivation were observed for the transient currents in HeLa cells cotransfected with plasmids pMSVR1 and pMSVR4 or with plasmid pMSVR4, respectively. Half time of recovery was 2.1 ± 0.5 s ($n = 3$) for currents in cotransfected HeLa cells and 7.3 ± 3.4 s ($n = 9$) for RCK4 channels. Whole-cell current recordings in bath solution containing 10 mM TEA.

similar to the ensemble currents measured in cell-attached macro-patches. The single channel current amplitude was 0.67 ± 0.1 pA ($n = 5$) at 0 mV (Fig. 4e,f), which is significantly larger ($P < 0.001$) than currents mediated by RCK4 channels (0.4 ± 0.06 pA, Fig. 4d,f) but similar to currents mediated by RCK1 channels (0.73 ± 0.02 pA, Fig. 4c,f). The shape of the open-channel current-voltage relation was linear for channels in

coinjected oocytes whereas that of RCK4 channels was super-linear, confirming the results seen with instantaneous whole-cell currents (Fig. 2b).

In conclusion, our results suggest that coexpression of cloned RCK1 and RCK4 subunits in HeLa cells and *Xenopus* oocytes results in assembly of heteromultimeric RCK1, 4 channels with properties distinct from those of the homomultimeric channels. Currents mediated by RCK1 channels do not inactivate in the millisecond time range and are sensitive to DTX and TEA. Currents mediated by RCK4 channels inactivate rapidly, but are practically insensitive to DTX and TEA. The RCK1, 4 channels inherit properties from both RCK1 and RCK4 subunits mediating transient currents that are sensitive to DTX and TEA. The RCK1, 4 channels have a single-channel conductance that resembles RCK1 channels but their gating resembles RCK4 channels. Coexpression of RCK genes and the assembly of heteromultimers from RCK subunits could be a further mechanism generating functional diversity of voltage-activated K^+ channels in the mammalian central nervous system. □

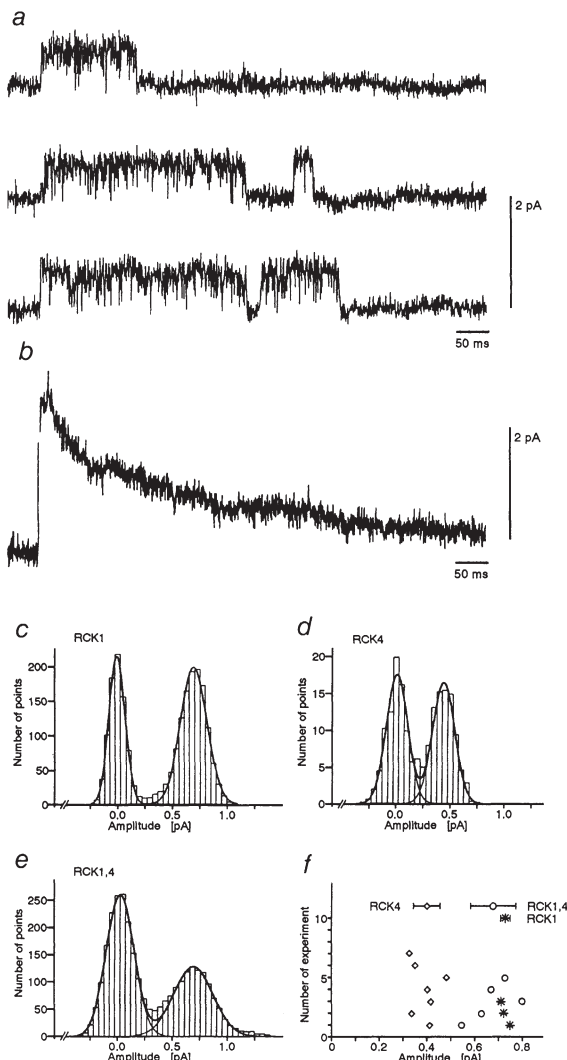


FIG. 4 Single-channel currents. *a*, Three traces of single-channel currents recorded in response to voltage steps from -70 to 0 mV under 10 mM TEA. Oocyte coinjected with RCK1- and RCK4-specific cRNA. All records were corrected for capacitive and leak currents by subtracting an average of 25 records in which no channel openings occurred. Records were filtered at 1 kHz (-3 dB) and sampled at 2 kHz. The mean amplitude of the single-channel currents is 0.67 pA. *b*, Average of 52 records from the same patch as shown in (*a*). One point caused by the capacitive artefact has been blanked out. Average current inactivates rapidly with a time constant of 188 ms. *c*, Histogram of the amplitude values from single-channel recording as in (*a*) but from an oocyte injected with RCK1 cRNA. Histograms of current amplitudes were formed from digitized records where only a single channel opened. A sum of two gaussians was fitted to the amplitude histograms. The difference between their means was taken as single-channel current amplitude. The value 0.0 pA, indicated on the x-axis, designates the level of the baseline. The single-channel amplitude was 0.71 pA. *d*, Histogram of the amplitude values from single-channel recording from an oocyte injected with RCK4 cRNA. The single-channel amplitude was 0.43 pA. *e*, Histogram of the amplitude values from the experiment shown in (*a*). *f*, Scatterplot of the amplitudes of single-channel currents in different patches as indicated on the y-axis (RCK1, *; RCK4, ◇; RCK1, 4, ○). Mean and standard deviation are given by horizontal bars through the corresponding symbols.

Received 2 January; accepted 27 April 1990.

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ACKNOWLEDGEMENTS. We thank Dr W. Stühmer for several of the basic ideas of this study, Dr T. Verdoorn and Dr R. Kass for reading the manuscript and U. Warke for cell-culturing. We also thank Dr Habermann for the gift of dendrotoxin and C. Busch for the fitting routines. This work was supported by the Leibniz-Förderprogramm of the Deutsche Forschungsgemeinschaft.

Effect of bleached rhodopsin on signal amplification in rod visual receptors

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BLEACHING of rhodopsin markedly desensitizes the vertebrate visual system during a subsequent period of dark adaptation^{1–6}. Previous studies have indicated an origin of bleaching desensitization in the visual pigment itself, but have not identified the mechanism of action. A candidate for the site at which desensitization is initially expressed is the activation of transducin (formation of T^*) on the rod disk membranes; this reaction directly involves rhodopsin in its photoactivated (R^*) form and mediates initial amplification of the visual signal (reviewed in refs 7–9). We have analysed the effect of bleaching on the sensitivity of a flash-induced light-scattering signal known to monitor the disk-based amplifier^{10–12}, and which has been established as specifically monitoring transducin activation¹³. We have recorded this signal from functioning retinal rods *in situ* ('ATR' signal)¹⁴ and find that bleaches inducing a pronounced, sustained loss in rod electrophysiological sensitivity do not alter the sensitivity of the ATR

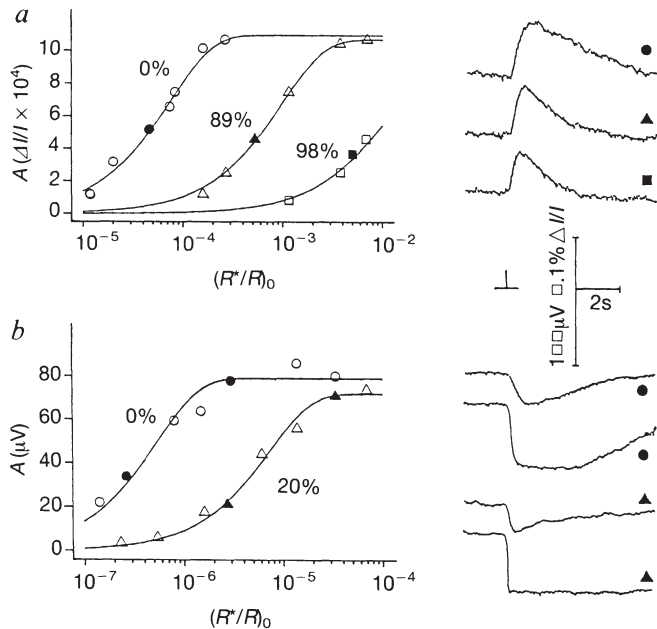


FIG. 1 *a*, Dependence of ATR amplitude (A) on stimulating flash intensity $[(R^*/R)_0]$. ATR signals recorded from a single retina before and after bleaching. $(R^*/R)_0$, fractional photoactivation of the total (unbleached retina) level of rhodopsin. Percentage bleach shown next to each curve. The condition of 89% bleaching was attained by four separate irradiations (cumulative bleach of 34%, 70%, 82.5% and 89%). Responses to test flashes presented after each bleach indicated the completion of ATR recovery (stabilization of the response) at about 90, 90, 45 and 30 min respectively after irradiation. ATR sensitivities (curves) determined from equation 1: $a_0 = 1.35 \times 10^6$ ($\circ$, $\bullet$); $a = 1.04 \times 10^3$ ($\triangle$, $\blacktriangle$); $a = 1.00 \times 10^2$ ($\square$, $\blacksquare$). Superfusion with low- Na^+ Ringer's at 20–22 °C (ref. 14). *b*, Amplitude versus intensity functions for ERG photoreceptor responses. Data obtained from a single retina before ($\circ$, $\bullet$) and after ($\triangle$, $\blacktriangle$) 20% bleaching. Post-bleach data obtained 1.2 h after irradiation, by which time the maximal ERG amplitude had fully recovered. Records analysed for amplitude at 1.3 s post-stimulus. ERG sensitivities (curves) determined from equation 1 (refs 30–31): $a_0 = 1.85 \times 10^5$ ($\circ$, $\bullet$); $a = 1.38 \times 10^5$ ($\triangle$, $\blacktriangle$). Waveforms correspond to amplitude data shown by filled symbols.

response after correction for reduced quantum catch. Our results indicate that the biochemical gain of the $R^* \rightarrow T^*$ transduction stage remains unchanged in the presence of bleached pigment and implicate a subsequent reaction as the first to show a sustained, bleaching-dependent gain reduction.

Bleached visual pigment, which arises on the deactivation and decay of R^* , maintains an electrophysiological desensitization greatly exceeding that due merely to the reduction in quantum capture by the bleached rod^{5,15–19}. The physiological effect of bleaching may be viewed as a reduction in the gain of phototransduction, i/R^* , where i is the photocurrent change generated by a weak test flash and R^* is the number of flash-activated rhodopsin molecules. Our experiments were designed to establish whether bleaching similarly depresses T^*/R^* , the gain prevailing at the level of transducin activation. ATR scattering signals ($\Delta I/I$ at 880 nm; scattering angle, $6^\circ < \Theta < 10^\circ$) were recorded from intact, isolated bovine retinas bathed in low- Na^+ Ringer solution¹⁴. In the initial phase of each experiment, the ATR amplitude against intensity function for the dark-adapted (unbleached) retina was obtained by presenting stimulating flashes (green light, 20 μs long) of varying intensity $[(R^*/R)_0]$; the probing flashes cumulatively bleached <2% of the rhodop-

sin. The photostimulator was then used to deliver a series of intense flashes that bleached a defined fraction of the rhodopsin.

As the isolated retina lacks the capacity to regenerate rhodopsin in significant quantity^{5,15–18}, the bleaching irradiation permanently reduced the efficiency of quantum capture by the rods. ATR responses to test flashes were monitored throughout the subsequent period of gradual recovery (which depends on the extent of bleaching, see Fig. 1), and a complete amplitude against intensity function was obtained on stabilization of the response. Multiple rounds of bleaching and post-bleach analysis of the ATR response were carried out in several preparations. Retinae maintained in aspartate-supplemented Ringer's²⁰ containing normal Na^+ concentration were similarly analysed for the effect of bleaching on the electroretinographic (ERG) photoreceptor response.

Bleaching of rhodopsin caused a sustained shift in the amplitude versus intensity function [A versus $(R^*/R)_0$] for the ATR scattering signal (Fig. 1*a*). Analysis of the ATR amplitude versus intensity data in terms of the function¹⁴

$$A/A_{\max} = \{1 - \exp[-a(R^*/R)_0]\} \quad (1)$$

yielded values for the sensitivity parameter, a , describing the

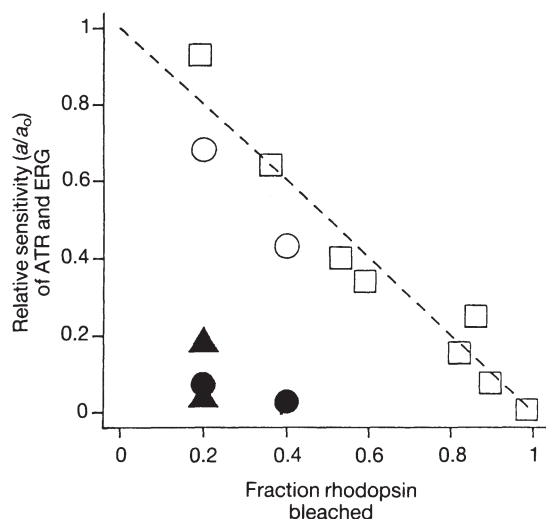


FIG. 2 Relative sensitivities (a/a_0) for the ATR ($\square$, $\circ$) and ERG ($\blacktriangle$, $\bullet$) versus fraction of rhodopsin bleached. $\square$, ATR data obtained in low- Na^+ Ringer's (six retinae). $\circ$, $\bullet$, a/a_0 data for ATR and ERG responses recorded simultaneously from the same retina (normal- Na^+ Ringer's); responses analysed for post-bleach values of ATR and ERG sensitivity were recorded within 30 min intervals centred at 75 min after the irradiations. $\blacktriangle$, ERG data obtained from two more retinae. ---, Quantum catch relation, $a/a_0 = [1 - (\text{fraction rhodopsin bleached})]$.

response function at a given fractional bleach. Over the entire bleaching range, decreases in the relative sensitivity of the ATR signal (a normalized to the dark-adapted value, a_0 ; open symbols in Fig. 2) were fully explained by the expected reduction in efficiency of quantum catch (dashed line in Fig. 2). By contrast, bleaching-induced desensitization of the ERG response (Fig. 1b; filled symbols in Fig. 2) greatly exceeded that due to reduced quantum catch and was comparable with previous electrophysiological results^{5,16-18}. Both the waveform and maximal amplitude ($A_{\max}$) of the ATR remained essentially constant with bleaching (Fig. 1). Among 10 determinations involving fractional bleaches of 0.19–0.98 (retinae described in Fig. 2), the ratio, ($A_{\max}$ after bleaching)/($A_{\max}$ of unbleached retina), was 1.1 ± 0.5 .

Operation of the disk-based amplifier in a given state of bleaching may be further characterized by the kinetics of ATR deactivation observed in a paired-flash experiment (conditioning and probing flash). In unbleached retinae, the delay period (T_d) preceding return of the ATR after a conditioning flash increases logarithmically with the conditioning flash intensity¹⁴. Consistent with a bleach-independent property of the processes governing ATR deactivation, values of T_d obtained before and after 50% bleaching of the retina (Fig. 3) showed a similar dependence on conditioning flash intensity after correction for quantum catch (Fig. 3, inset), and values of ATR recovery time (τ_r) determined before and after bleaching were generally similar (Fig. 3).

That substantial bleaching does not alter the photic sensitivity of the disk-based amplifier implies a constancy of operation of the amplifying step itself, namely, transducin activation. The evident preservation of $A_{\max}$ furthermore indicates that the size

of the transducin pool available for flash activation remains essentially undiminished on bleaching. Transmission of the visual signal through the rhodopsin/transducin system depends on multiple, membrane-localized biochemical events, beginning with the activation of rhodopsin and resulting in the production of GTP-activated transducin⁷⁻⁹. Furthermore, operation of the system is governed by the kinetics of deactivation of both R^* and T^* (refs 21, 22). As effective amplification must depend critically on the interplay of these events⁹, it is remarkable that the sensitivity of the system remains undiminished on the generation of a large quantity of bleaching product in the disk membrane.

Ca^{2+} and cyclic GMP, substances that undergo light-dependent concentration changes in reactions 'downstream' from transducin activation, are believed to have a role in rod adaptation²³⁻²⁵. The present observation (Fig. 2, circles) that ATR sensitivity and, thus, T^*/R^* gain remain constant upon marked desensitization of the ERG response is of particular interest in this regard, as this finding argues against a basis of bleaching desensitization in feedback by Ca^{2+} or cGMP to the $R^* \rightarrow T^*$ reaction. Properties of cGMP phosphodiesterase (PDE) activity in rod disk membrane preparations²⁶ also argue against the regulation of transducin activation by Ca^{2+} .

The apparent independence of bleaching desensitization and R^*/T^* gain raises the possibility of a branched pathway of signal transmission from the visual pigment³, in which R^* activates T (transduction branch) but bleached pigment directly influences a reaction subsequent to the formation of T^* (desensitization branch). However, the data do not exclude the alternative possibility that the desensitizing signal from bleached pigment involves maintained, dark activation of a small fraction of T . As the molar quantity of T in the rod exceeds that of PDE by about 10-fold⁷⁻⁹, dark activation of a small fraction of the T (a fraction without detectable effect on $A_{\max}$) could activate a large fraction of the PDE in darkness. By reducing the flash-activatable pool of PDE, this non-zero dark level of T^* could reduce the gain of flash-induced PDE activation and thus reduce i/R^* (ref. 27). Both possibilities are consistent with transmission of an active, 'dark light' signal from bleached pigment in the absence of a probing flash^{3,19,28-29}.

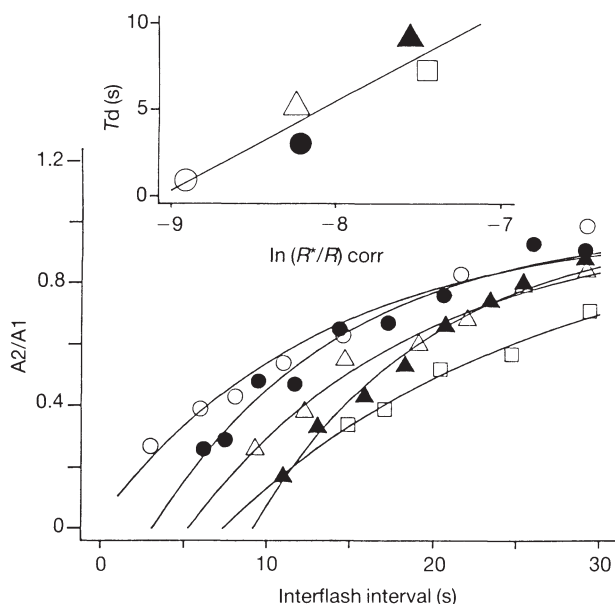


FIG. 3 Deactivation of ATR response examined by a paired-flash procedure¹⁴. Equal flash intensities [$(R^*/R_0)_0$] for the first (conditioning) and second (probing) flashes of a given pair. A_1 and A_2 are amplitudes of ATR responses to first and second flash, respectively. Recovery (A_2/A_1) plotted against interflash interval. Filled and open symbols: data obtained, respectively, before and after a 50% bleach. $(R^*/R_0)_0 = 2.70 \times 10^{-4}$ (●, ○); 5.25×10^{-4} (▲, △); 11.55×10^{-4} (□). Smooth curves: analysis of data in terms of: $A_2/A_1 = \gamma_1 \{1 - \exp[-(t - T_d)/\tau_r]\} + \gamma_2$, where T_d = delay period; τ_r = recovery time; γ_1 = constant; γ_2 = constant¹⁴. For all curves except (○), $\gamma_1 = 1$ and $\gamma_2 = 0$. τ_r (s): 11.5 (●); 10.8 (▲); 13.5 (○); 13.7 (△); and 18.9 (□). Inset, relation between T_d and $[\ln(R^*/R_0)_{\text{corr}}]$ before (filled symbols) and after (open symbols) 50% bleach. $(R^*/R_0)_{\text{corr}}$ = flash intensity corrected for quantum catch = $(R^*/R_0)_0 (1 - (\text{fraction rhodopsin bleached}))$.

Received 6 October 1989; accepted 20 March 1990.

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ACKNOWLEDGEMENTS. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the US Public Health Service.

A role for clonal inactivation in T cell tolerance to Mls-1^a

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CLONAL deletion plays a major part in the maintenance of natural self-tolerance in both normal¹⁻⁵ and transgenic⁶⁻⁹ mice. Self antigens that are expressed in the thymus result in the physical elimination of autoreactive thymocytes at a particular stage in their development. For example, the majority V β 6- and V β 8.1-bearing T cells that recognize the minor lymphocyte-stimulating antigen, Mls-1^a (ref. 10), are clonally deleted in the thymuses of normal mice and transgenic mice expressing Mls-1^a (refs 2, 3, 9). In contrast, a very different mechanism of tolerance involving the functional inactivation, but not elimination, of autoreactive cells, termed clonal inactivation or clonal anergy, has been implicated in some experimentally manipulated systems of tolerance¹¹⁻¹⁵. To test further the mechanisms involved in self-tolerance, we have generated transgenic mice expressing a V β 8.1 beta chain on >95% of peripheral T cells and have tested tolerance to Mls-1^a in these mice. Surprisingly, a significant fraction of the CD4⁺ peripheral cells that survived deletion were non-responsive *in vitro* to any stimulus tested. Naturally occurring tolerance to a self antigen expressed in the thymus can thus be mediated by clonal anergy, as well as by clonal deletion.

T-cell recognition of antigen-MHC is mediated by the variable elements of the T-cell antigen receptor (TCR) alpha (V α J α) and beta (V β D β J β) chains¹⁶. But recognition of superantigens is dominated by contribution from the V β element, so responsive T cells can be identified on the basis of TCR V β expression alone^{1-5,17-19}. This approach has been used in the study of self-tolerance to a variety of self antigens and has demonstrated a major role for clonal deletion as a mechanism of natural T-cell tolerance¹⁻⁵. But although clonal anergy has been implicated as a mechanism in adult-induced tolerance^{13,14}, in transgenic (TG) mice where molecules causing tolerance are expressed on non-haematopoietic tissues^{11,12}, or in certain P to F₁ radiation chimaeras¹⁵, the importance of clonal anergy in naturally occurring tolerance remains unclear.

A well characterized *in vivo* system of tolerance is that mediated by the minor lymphocyte stimulating antigen, Mls-1^a, which is recognized by almost all V β 6-, V β 8.1-, and V β 9-bearing T cells^{2,3,19}. Naturally occurring tolerance of V β 8.1- and V β 6-bearing T cells to Mls-1^a has been shown to be mediated by clonal deletion in the thymus^{2,3,9}. Likewise, V β 6⁺ T cells are deleted as a result of experimentally induced neonatal tolerance²⁰. In contrast, immunization of adult mice with Mls-1^a does not result in deletion of V β 6⁺ T cells^{13,14}, but seems to render the cells non-responsive, or anergic¹⁴. Clonal anergy of V β 6 cells has also been described in P to F₁ chimaeric mice in which the expression of Mls-1^a in the thymus has been experimentally manipulated¹⁵.

T-cell receptor β -chain transgenic (β TG) mice were produced with a V β 8.1 D β 2 J β 2.3 C β 2 genomic construct that included the TCR enhancer sequences²¹. The founder mouse was H-2^k and Mls-1^b. The transgene was expressed on >95% of both the CD4⁺ and CD8⁺ populations of peripheral T cells (Fig. 1b, e), exhibited allelic exclusion and was expressed in a tissue-specific manner (data not shown). The original T-cell hybridoma from

which the transgene was cloned, 3DT-52.5 (ref. 22), was strongly Mls-1^a-reactive. Therefore, it was surprising that analysis of 29 T-cell hybridomas generated from these transgenic mice showed that only 41% were capable of recognizing Mls-1^a, in spite of the fact that all the hybridomas expressed both the V β 8.1 transgenic β -chain and CD4, and responded to stimulation with immobilized anti-V β 8 antibodies (data not shown). We conclude that the particular VDJ combination expressed in the transgenic β -chain is, by itself, not strongly Mls-1^a-reactive, but is dependent upon the contribution from the alpha chain with which it associates. So, the T-cell repertoire of these transgenic mice includes a spectrum of Mls-1^a-reactivities.

The founder mouse (H-2^k, Mls-1^b) was crossed with CBA/Ca (H-2^k, Mls-1^b) and CBA/J (H-2^k, Mls-1^a) mice to assess the mechanism of tolerance to Mls-1^a. Both the Mls-1^a β TG and the Mls-1^b β TG mice expressed high frequencies of V β 8.1⁺ T cells within the CD4⁺ and CD8⁺ populations (Fig. 1b, c, e, f). In addition, receptor density and accessory molecule densities of CD4⁺ and CD8⁺ T cells from both types of animals were equivalent to normal mice (Fig. 1a, d). Also, the number of thymocytes and peripheral T cells in the two types of mice were comparable, and, in agreement with the observations of others^{8,9}, we did not find evidence for accelerated thymocyte maturation in our β TG mice (data not shown), as has been observed in the α β TG mice⁶. There was some evidence of an effect of Mls-1^a expression on CD4⁺ cells however. First, the percentage of CD4⁺ cells expressing the V β 8.1 transgene was lower in Mls-1^a β TG mice. The Mls-1^b β TG mice consistently expressed ~95% TG⁺ CD4⁺ T cells, whereas expression in the Mls-1^a β TG mice ranged between 85-92% in 2-3 month-old mice. Transgene expression in the CD8⁺ population of T cells was >95% for both types of mice. Second, analysis of the CD4-CD8 distribution of peripheral T cells showed a dramatic and consistent swing toward CD8 expression in the Mls-1^a β TG mice (data not shown).

Failure to respond to *in vitro* stimulation with Mls-1^a-bearing spleen cells showed that the Mls-1^a β TG mice were indeed tolerant to Mls-1^a (Fig. 2a). We then looked in the thymus for

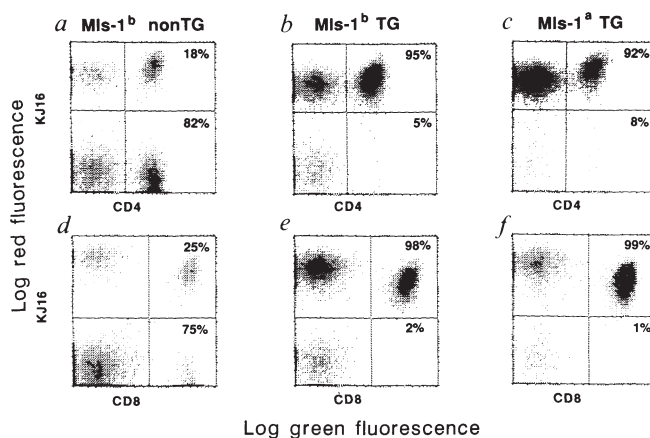
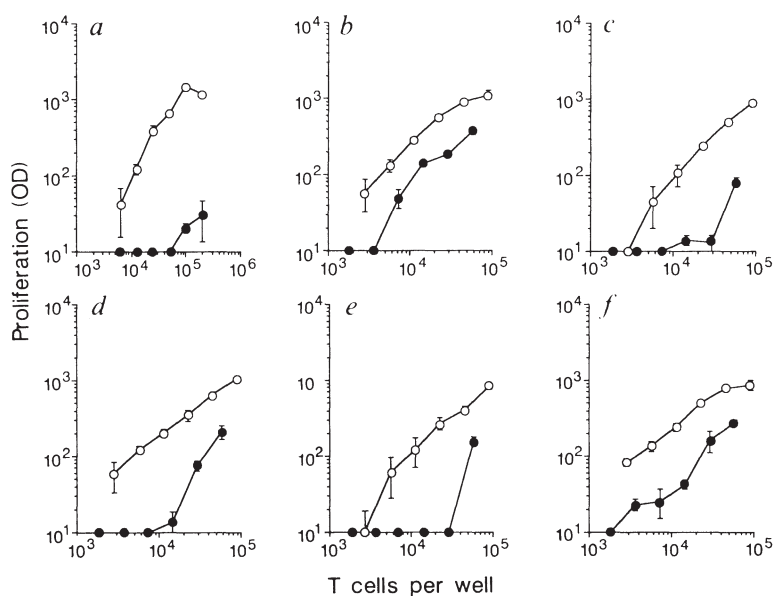


FIG. 1 Expression of the transgenic V β 8.1⁺ β chain in the CD4⁺ and CD8⁺ populations of peripheral T cells in transgenic(TG) and non-transgenic mice. Nylon wool-purified peripheral T cells from Mls-1^b (CBA/Ca) non-transgenic mice (a, d), Mls-1^b β TG mice (b, e), and Mls-1^a β TG mice (c, f) were analysed by two-colour fluorescence for V β 8 expression in the CD4⁺(a, b, c) and CD8⁺(d, e, f) populations of T cells using biotin-conjugated KJ16 (ref. 23) (V β 8.1, 8.2) followed by phycoerythrin-avidin, and directly-fluorescinated anti-CD4 (GK1.5) or anti-CD8 (53.6) antibodies. Peripheral T cells from the transgenic mice failed to stain with F23.2 (ref. 30) (V β 8.2), confirming that all the T cells staining with KJ16 expressed the V β 8.1⁺ TCR (data not shown). The percentages refer to the per cent of KJ16⁺ and KJ16⁻ T cells within the CD4⁺ (top panel) or CD8⁺ (bottom panel) populations of T cells.

FIG. 2 CD4⁺ peripheral T cells from Mls-1^a-expressing β TG mice are tolerant to Mls-1^a and non-responsive to a variety of *in vitro* stimuli. *In vitro* proliferation of CD4⁺ T cells from Mls-1^a (●) and Mls-1^b (○) β TG mice was measured in response to Mls-1^a, expressed by 5×10^5 mitomycin C-inactivated CBA/J spleen cells (a). b–f. Responses of CD4⁺ T cells to stimulation (in the presence of 5×10^5 mitomycin-treated Mls-1^b spleen cells) with immobilized anti-V β 8 antibodies (KJ16; ref. 23), 10 μ g ml⁻¹ *Staphylococcus enterotoxin* B, immobilized anti- $\alpha\beta$ TCR antibodies (597; ref. 25), immobilized anti-CD3_ε antibodies (145-2C11; ref. 26), and 4 μ g ml⁻¹ concanavalin A, respectively. Cultures (250 μ l) were incubated for 3–6 days and proliferation was measured by a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay³¹. The CD4⁺ populations of cells were purified by panning on anti-CD8 (53.6)-coated plates. Each point represents the mean of triplicate determinations $\pm$ s.e.m. The Mls-1^b β TG CD4⁺ T cells were 97% V β 8.1⁺ and the Mls-1^a β TG CD4⁺ T cells were 78% V β 8.1⁺. OD, Absorbance at 570 nm.



direct evidence of clonal deletion by comparing the number of mature V β 8.1⁺ thymocytes in the Mls-1^b and Mls-1^a β TG mice. Whereas in normal Mls-1^a mice² and Mls-1^a β TG mice⁹, most V β 8.1⁺ thymocytes are eliminated before the mature, single positive stage of thymocyte development, we found only a modest reduction (mean = 20%) in the number of mature, V β 8.1⁺ thymocytes in our Mls-1^a β TG mice. We do not think that this was due to a defect in the expression of Mls-1^a in these mice, because analysis of non-transgenic littermates indicated that Mls-1^a is expressed early and is capable of deleting most V β 6⁺ and V β 8.1⁺ mature thymocytes (data not shown). Rather, clonal deletion is probably difficult to demonstrate in these Mls-1^a β TG mice because all of the thymocytes are V β 8.1⁺. Therefore, the rapid repletion of the thymic compartments with non-Mls-1^a-reactive V β 8.1⁺ thymocytes could mask deletion. The measure of reduction in mature thymocyte number is probably an underestimate of the degree of clonal deletion in these mice.

We initially assumed that the CD4⁺V β 8.1⁺ T cells in the periphery of the Mls-1^a β TG mice were T cells incapable of recognizing Mls-1^a, owing to pairing with a non-permissive alpha chain. However, this was not the only explanation for the high number of peripheral CD4⁺ cells that survived deletion. Analysis of the *in vitro* reactivity of T cells from Mls-1^a β TG mice showed that a significant proportion of the CD4⁺ cells were non-responsive to any stimulus. First, we tested purified populations of CD4⁺ and CD8⁺ T cells for reactivity with immobilized anti-V β 8 antibodies (KJ16, ref. 23) and *Staphylococcus enterotoxin* B (SEB), a superantigen that stimulates a high proportion of CD4⁺ and CD8⁺ V β 8.1-bearing T cells^{17,24}. Comparison of reactivity to V β 8 antibodies (Figs 2b, 3a, b) and SEB (Figs 2c, 3c, d) by CD4⁺ and CD8⁺ T cells from Mls-1^a- and Mls-1^b- β TG mice revealed a two- to three-fold reduction in responsiveness to both stimuli, that was predominant in the CD4⁺ population of T cells (Fig. 3a–d). CD4⁺ T cells from the Mls-1^a β TG mice also demonstrated reduced responsiveness when stimulated with anti TCR $\alpha\beta$ antibodies²⁵, anti-CD3_ε antibodies²⁶, and concanavalin A (conA), a T-cell mitogen (Fig. 2d–f). We have evidence, therefore, that a significant fraction of the CD4⁺ population of T cells in the Mls-1^a β TG mice is anergic. About one third of the mice fail to demonstrate *in vitro* anergy. We do not know if this inconsistency is due to genetic (the mice are not inbred), environmental or physiological differences. Peripheral T cells and thymocytes from these mice show no difference in number or cell surface phenotype from

the anergic mice and we do not yet understand how tolerance is achieved in these non-deleted mice.

Analysis of reactivity of mature thymocytes from the anergic mice failed to reveal evidence of non-responsiveness (Fig. 4). This indicates either that the anergy is induced in the periphery, or that cells which become anergic in the thymus do not accumulate, but quickly leave from the thymus. If anergy is induced in the thymus, it is at a relatively late stage of thymocyte development, after clonal deletion which acts on the immature, double-

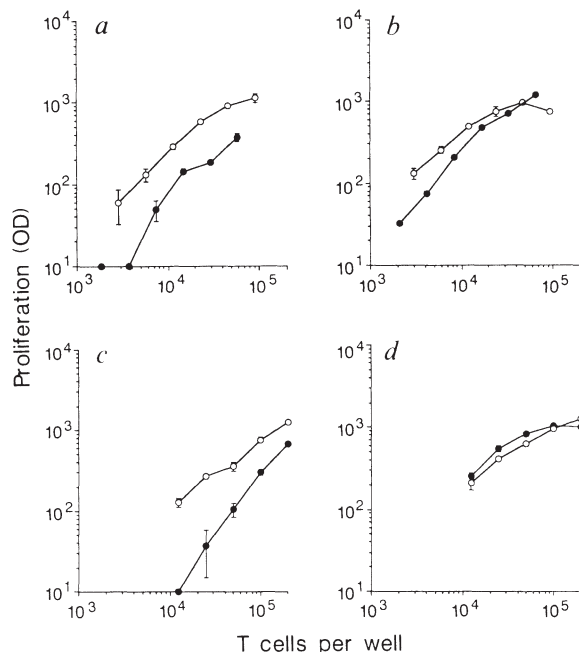


FIG. 3 Non-responsiveness of peripheral T cells from Mls-1^a-expressing β TG mice is confined to the CD4⁺ population. CD4⁺ (a, c) and CD8⁺ (b, d) peripheral T cells from Mls-1^a β TG (●) and Mls-1^b β TG (○) mice were purified by panning on anti-CD8 (53.6)- and anti-CD4 (GK1.5)-coated plates, respectively, and tested for their proliferation to immobilized anti-V β 8 antibodies (a, b) and SEB (c, d), as described in Fig. 2. Each point represents the mean of triplicate determinations $\pm$ s.e.m., except the Mls-1^a β TG data in b, which represents the average of duplicate points.

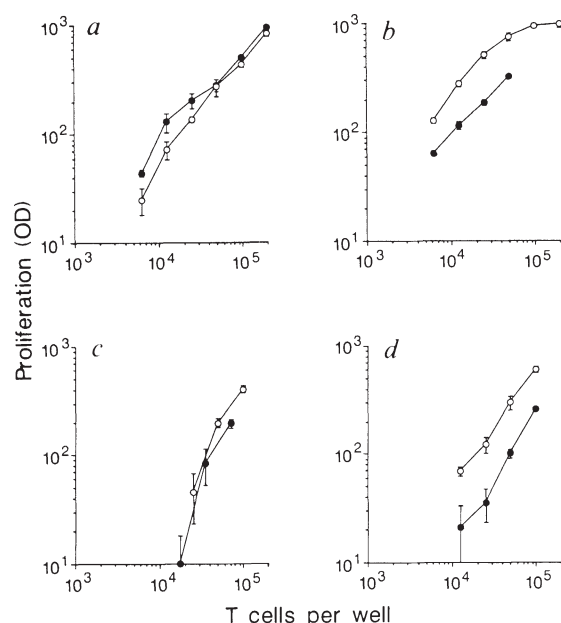


FIG. 4 Mature thymocytes from Mls-1^a β TG mice are not anergic. *In vitro* proliferation of mature CD4⁺ thymocytes (a, c) and CD4⁺ peripheral T cells (b, d) from Mls-1^a β TG (●) and Mls-1^b β TG (○) mice in response to stimulation with immobilized anti-V β 8 antibodies (a, b) and SEB (c, d) was measured as described in the legend to Fig. 2. Mature thymocytes were purified by panning on anti-CD8-coated plates, which depleted the immature CD4⁺CD8⁺ thymocytes, as well as the mature CD8⁺ thymocytes. The resulting populations were >60% CD4⁺CD8⁻. Each point represents the mean of triplicate determinations $\pm$ s.e.m.

positive (CD4⁺CD8⁺) thymocytes, resulting in the deletion of both CD4⁺ and CD8⁺ T cells^{3,6,7-9,27}. But only CD4⁺ cells (those that would recognize Mls-1^a in the context of class II MHC) become anergic, indicating that this event occurs either at a later, single positive stage of thymocyte development or on CD4⁺ T cells in the periphery.

We conclude that V β 8.1⁺ T cells in these transgenic mice have three possible fates. The Mls-1^a-reactive cells are subject to two mechanisms of tolerance, clonal deletion in the thymus and clonal anergy in the periphery. The cells that cannot recognize Mls-1^a, presumably due to pairing with a non-permissive α -chain, are unaffected by either mechanism of tolerance and constitute the population of normally reactive cells in the periphery. This accounts for the fact that peripheral anergy only results in a reduction in the response of peripheral T cells, rather than complete ablation.

Why are all the V β 8.1⁺ Mls-1^a-reactive cells not deleted, as has been observed both for normal mice² and another V β 8.1 TG mouse⁹. The answer may lie in the affinity of the TCR for Mls-1^a. The β chain used in these studies is not inherently strongly Mls-1^a-reactive, and is dependent upon the α -chain for Mls-1^a reactivity, possibly resulting in a spectrum of affinities. Perhaps the high-affinity clones are deleted, whereas the low-affinity clones escape deletion and are subject to peripheral mechanisms of tolerance, such as clonal anergy. Peripheral anergy could serve as a minor, back-up mechanism of tolerance in normal mice, which is amplified in the transgenic mouse. This theory remains to be tested.

We do not yet understand the mechanism of anergy. The non-responsive cells in the periphery exhibit a normal cell surface phenotype, in that neither TCR nor CD4 molecules are down-regulated, unlike the situation found in some other B-cell²⁸ and T-cell⁶ transgenic models for tolerance. Furthermore, the non-responsiveness cannot be overcome by signalling directly

with anti-CD3_ε antibodies, implying that the defect is not in the coupling of TCR and CD3 as described for tolerance induction in the thymus²⁹. The mechanism of T-cell anergy in the periphery of the Mls-1^a-expressing β TG mice is under investigation. □

Received 4 December 1989; accepted 21 March 1990.

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Induction of sweat glands by epidermal growth factor in murine X-linked anhidrotic ectodermal dysplasia

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TABBY (*Ta*)¹, a murine X-linked mutant gene, produces a syndrome of ectodermal dysplasia including anhidrosis² (absence of sweat glands). Development of sweat glands is related to that of dermal ridges (dermatoglyphics)³ and abnormal ridges may be associated with absence of sweat glands⁴ in the human syndrome of hypohidrotic ectodermal dysplasia (HED)⁵. We have found that dermal ridges occur in normal mice but are lacking in *Ta* mutants. Previously we showed that epidermal growth factor (EGF) reverses delayed eyelid opening and incisor eruption in *Ta* mice⁶. We now report that EGF induces development of dermal ridges and functional sweat glands in *Ta/Y* hemizygotes, indicating a role in mammalian morphogenesis⁷. *Ta* seems to be genetically homologous to human X-linked HED², as *Ta* maps close to loci homologous to linkage markers of HED⁸ and the two syndromes share many traits, including absence of all or most sweat glands². Absence of these glands causes hyperpyrexia, a clinical emergency in infants with HED; reversal of the trait in the mouse homologue of the disease indicates that an important genetically determined congenital defect in humans may become treatable.

We used a *Ta*-bearing congenic line of the inbred strain DCH (ref. 9). Animals were studied for dermatoglyphics and ability

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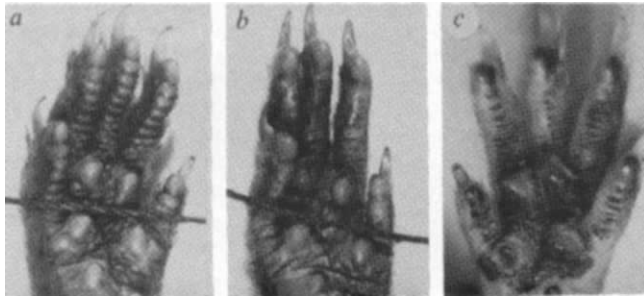


FIG. 1 Dermatoglyphics of mice (magnification approximately $\times 7$), following fixation and staining in Bouin's fixative (a and b) or inking (Footprinter Ready Rolled ink pad, Hollister Incorporated) (c). Paws are held in position by string (a, b). a, Ridges of volar surface of normal, 45-day $+/Y$ male. b, Volar surface of 45-day Ta/Y hemizygote (littermate of $+/Y$ shown in a) showing absence of ridges. c, Ridges seen in Ta/Y male treated with EGF (Collaborative Research), $4 \mu\text{g}$ per g bodyweight by daily subcutaneous injection, $100 \mu\text{g}$ per 0.25 ml, from birth to day 30 of postnatal life. EGF used is 99% pure, and gives a single band of relative molecular mass (M_r) 6,100 on SDS-PAGE (R. Gilbert, personal communication).

to sweat, and histologically for the presence of sweat glands, as described in Figs 1–3. Some Ta/Y mutants and $+/Y$ wild-type littermates were injected with EGF from birth, and control animals of both genotypes were injected with saline.

All 10 normal adult males and 10 normal adult females examined had dermal ridges (Fig. 1a). Ridges were absent at birth in ~ 20 normal neonates studied but developed by day 7. All 10 adult Ta/Y mutants lacked ridges (Fig. 1b). All eight Ta/Y hemizygotes injected with EGF for 7 or more days postnatally developed dermal ridges by day 7 (Fig. 1c). The ridges were smaller, however, and ridge counts were lower than in normal mice. Mutants injected with saline showed no ridges (Fig. 2c) even after inking (data not shown). Normal mice have palmar and volar sweat glands, whereas Ta/Y mutants lack them². Normal mice injected with EGF also sweat (Fig. 2a). All three mutants injected with EGF every day from birth to day 30 (prepuberty) and examined for sweating, sweated when tested on days 35 (puberty) and 45 (young adulthood) (Fig. 2b), albeit less profusely than usual². One of the three injected mutants sweated despite receiving no adrenaline, although at only two very small apparent sweat-pore sites.

Histological serial sections through volar surfaces of paws (~ 200 sections per animal) were studied as previously². In both

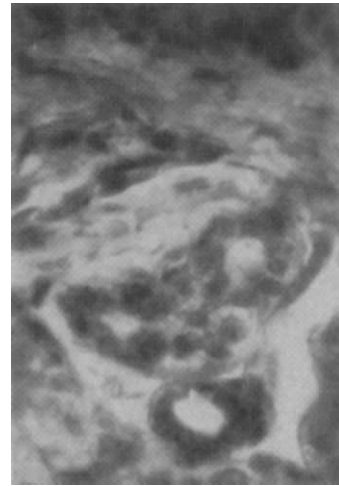


FIG. 3 Histological serial section (from demineralized material prepared as in ref. 2) through skin of volar surface of Ta/Y hemizygote treated with EGF as described (Fig. 1c). Sweat gland ducts (lower part of picture) are lying deep to epidermis (top). No sweat glands were seen in untreated Ta/Y animals. Magnification, $\times 500$.

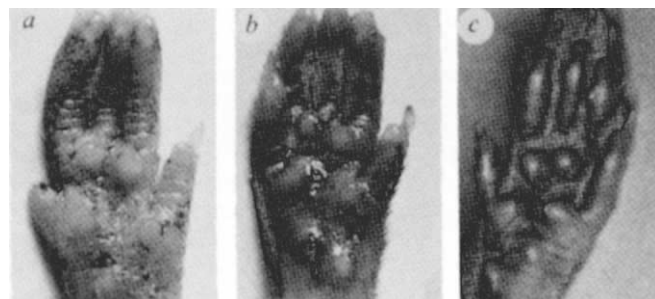


FIG. 2 Demonstration of sweating. Following sympathomimetic injection of adrenaline, sweating is detected as black dots by starch-iodine technique of Hayashi³² (magnification $\times 7$). a, Normal 45-day $+/Y$ male treated with EGF as described (Fig. 1c). b, Ta/Y hemizygote treated with EGF as above, showing sweating. c, Saline-treated Ta/Y hemizygote, showing total anhidrosis (as do untreated Ta/Y mice²) and absence of ridges.

EGF-treated mutants examined histologically (one of the above-mentioned animals and one other treated similarly), sweat glands were seen (Fig. 3). Two saline-injected and two uninjected mutants tested after adrenaline injections showed no evidence of sweating (Fig. 2c), nor were sweat glands seen in the two saline-injected animals studied histologically. Cell clusters occasionally seen in these could have been remnants of sweat gland rudiments which failed to develop. Untreated Ta/Y mice previously studied² did not sweat nor were sweat glands seen. The present results were obtained using only one dosage regime; other schedules, and possibly combination of EGF with a synergist such as retinoic acid¹⁰, might improve the result.

Studies *in vitro* indicate that EGF has a role in the development of the breast¹¹, a modified sweat gland, and possibly in other structures, the development of which involves mesenchymal-epithelial interactions, such as the lung¹², and, at least in some situations, hair follicles and tooth germs¹³. EGF is also secreted by glandular and other structures derived from mesenchyme-epithelium, including salivary glands¹⁴, Brunner's glands of the small intestine¹⁵, the breast¹⁶ and sweat glands¹⁷, and its precursor is evidently produced in tooth germs¹⁸. We propose that in the development of at least some such structures, production of EGF is induced at the site of development by mesenchymal-epithelial contact. The primary role of the EGF so produced would be autocrine promotion of morphogenesis of the structure and, in some cases, possibly development of neighbouring structures through gradient and 'field effects'¹⁹. In some cases, such as salivary glands, sweat glands and breast, EGF secretion would continue, after development is completed, as a secondary functional trait. A similar autocrine role in morphogenesis has been proposed for Mullerian Inhibiting Factor, which is produced by Sertoli cells and induces their development²⁰. An autocrine role for EGF in the development of malignancy has been proposed²¹. Different selective forces might maintain EGF secretion, for example in milk for morphogenesis of the infant and in saliva (and possibly sweat) for wound healing²².

We hypothesize that Ta acts, at least in part, by interfering with the proposed developmental and functional roles of EGF. Ta retards or suppresses the development and function of sweat glands², tooth germs²³, hair follicles¹, respiratory mucous glands (S.R.B. and K. Arnold, unpublished results), and various other

murine glands²⁴, in particular the EGF-producing granular convoluted tubules of the submandibular gland of the mouse²⁵, the richest known source of EGF.

Proteins highly homologous with EGF have central roles in development in *Caenorhabditis elegans*²⁶, *Drosophila*²⁷ and the sea urchin²⁸. The gene for the EGF receptor is highly homologous with the oncogene *c-erb-B* (ref. 29). Our finding may have general significance in the context of genetic control of mammalian development.

Neither *Ta* nor the gene for HED has been sequenced, and true molecular homology is therefore not yet proven. Nevertheless, there is compelling evidence that they constitute an example of Ohno's law of mammalian X-chromosomal evolutionary conservation³⁰. First, there is a striking overlap of phenotypic traits³¹. Second, both genes map close to the loci for phosphoglycerate kinase and X-chromosomal activation⁸ (*Xce* in the mouse). Consequently, our results may be relevant to future therapeutic applications in HED. □

Received 9 October 1989; accepted 20 March 1990.

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ACKNOWLEDGEMENTS. This work was aided by a grant from the Natural Sciences and Engineering Research Council of Canada (S.R.B.). We thank Dr R. P. Erickson, (Department of Human Genetics, University of Michigan), for comments on the manuscript.

Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap

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EUKARYOTIC cellular mRNAs have a 5' cap structure (m⁷GpppX) that facilitates binding to ribosomes and is required for efficient translation^{1–3}. A specific initiation factor, eIF-4F, mediates the function of the cap^{4–6} and consists of three subunits^{7–10}, one of which, eIF-4E, binds the cap. This subunit is present in limiting amounts in the cell^{11–13}, and is thought to be regulated by phosphorylation^{12–16}: decreased phosphorylation of eIF-4E following various treatments correlates with a decrease in cellular translation rate. These observations suggest that eIF-4E lies on the mitogenic signal transduction pathway, and we reasoned that overexpression of eIF-4E might profoundly affect cellular growth properties. We report here that overexpression of eIF-4E in NIH 3T3 and Rat 2 fibroblasts causes their tumorigenic transformation as determined by three criteria: formation of transformed foci on a monolayer of cells; anchorage-independent growth; and tumour formation in nude mice.

To test the hypothesis that overexpression of eIF-4E will result in alteration of cell growth, we infected NIH 3T3 cells with a retroviral expression construct¹⁷, pMV7-4E, which contained the full length eIF-4E sequences (Fig. 1a). Controls were NIH 3T3 cells infected with the pMV7 expression vector lacking eIF-4E cDNA sequences, or containing an eIF-4E complementary DNA fragment (pMV7-4E (Ala)) whose serine 53 codon was mutated to alanine. Serine 53 is the main phosphorylation site in eIF-4E (ref. 18), and its phosphorylation is thought to activate eIF-4E function¹⁹. Overexpression of the serine-mutated eIF-4E cDNA served as a negative control, as it should encode

a non-functional eIF-4E. Thirteen neomycin (G418)-resistant colonies were isolated and characterized. A detailed study of two clones: pMV7-4E (C1) and (C2) is presented (all the other clones showed similar properties to (C1) and (C2)). The remaining G418-resistant colonies were pooled and grown into mass culture (termed pMV7-4E(P)).

The level of expression of eIF-4E RNA in the different G418-resistant clones was examined by northern blot analysis. A representative analysis using poly(A)⁺ messenger RNA is shown in Fig. 1b. NIH 3T3 cells express an endogenous ~1.6-kb RNA molecule that hybridizes to the eIF-4E cDNA probe. This hybridization signal is too faint to be seen in lane 1 (Fig. 1b), and could only be detected after a longer exposure of the autoradiogram (Fig. 1c, lane 1, arrowhead). A similar signal was obtained in NIH 3T3 cells infected with the pMV7 virus (data not shown). In 3T3-pMV7-4E(P), expression of the ~1.6 kb eIF-4E mRNA is vastly increased (~50–100-fold). This mRNA is most probably derived from the exogenous eIF-4E cDNA, as it contains a polyadenylation site in its 3' untranslated region. In addition, an eIF-4E mRNA species of ~5.0 kb is expressed in large amounts (Fig. 1b, lane 2, arrow). The latter RNA species probably results from readthrough of the polyadenylation signal in eIF-4E cDNA and termination in the 3' Moloney murine leukaemia virus (MuLV) long terminal repeat. G418-resistant cell lines expressed both eIF-4E mRNA species at varying levels. The level of expression of the two mRNA species in 3T3-pMV7-4E (Ala) was approximately 15–30% of that in the two clones (C1) and (C2) (compare lane 5 with 4 and 3).

The amount of eIF-4E protein in the different clones was determined by western blotting (Fig. 1d) and immunoprecipitation analysis (data not shown). The amount of eIF-4E in cells infected with pMV7-eIF-4E or pMV7-eIF-4E (Ala) was increased 5–8-fold (Fig. 1d; compare lanes 2–5 with 1). The increase in the amount of eIF-4E protein was smaller than the increase in the amount of mRNA, suggesting a post-transcriptional down-regulation of eIF-4E expression. Levels of β -actin were increased by only ~1.5 fold in the infected cells (compare lanes 2–5 with 1). In this experiment the levels of eIF-4E protein in cells overexpressing eIF-4E (Ala) are slightly lower (~10–20%) than in cells overexpressing wild-type eIF-4E. Several other cell lines overexpressing lower levels of wild-type eIF-4E

relative to eIF-4E (Ala) still showed transformation properties similar to C1 and C2 (data not shown). We also found a higher level of phosphorylation on overexpressed wild-type eIF-4E, compared with the levels in NIH 3T3 cells and 3T3-pMV7-4E (Ala) (data not shown).

The growth properties of the cell lines overexpressing eIF-4E were markedly different from those of the parent NIH 3T3 cells. The doubling time of the three different cell lines overexpressing eIF-4E was reduced from 27 h in NIH 3T3 or 3T3-pMV7 to 20–22 h (Fig. 2, Table 1). Overexpression of eIF-4E (Ala) did not enhance cell growth but extended the doubling time by ~10–15%. This change is statistically significant ($P < 0.02$), and might reflect a *trans*-dominant negative effect of the serine-mutated eIF-4E. Cells overexpressing eIF-4E also grew to a higher density (1.5–2-fold) relative to controls (Fig. 2, Table 1).

Cell lines overexpressing eIF-4E showed changes in morphology (becoming spindle-shaped) and an increase in refractility, suggesting a transformed phenotype. When 3T3-pMV7-4E (P), (C1) and (C2) cell lines were grown to confluence, transformed foci formed on the monolayer (Fig. 3a, b). Transformed foci did not form with NIH 3T3, 3T3-pMV7 or 3T3-pMV7-4E (Ala) (Fig. 3a, b). Transformed foci also formed without pre-

vious selection for neomycin resistance (an average number of 500 foci per 1×10^5 cells after 14 days; data not shown).

In addition, cells overexpressing eIF-4E acquired the ability to grow in soft agar, a property that often correlates with tumorigenicity in rodent cells²⁰. Figure 3c shows macroscopic colonies (0.4 mm–1 mm in diameter) formed with 3T3-pMV7-4E (P), (C1) and (C2). Similar sized colonies were observed with the other overexpressing eIF-4E clones (data not shown), but not with NIH 3T3, nor with 3T3-pMV7, and, most significantly, not with 3T3-pMV7-4E (Ala). Efficiency of colony formation in soft agar was ~20% (Table 1), which is similar to the efficiency of colony formation of cell lines transformed with other oncogenes (ref. 21).

When cells (2.5×10^6) from cell lines overexpressing eIF-4E were injected into nude mice, tumours developed within a relatively short latent period (10–15 days; Table 1). Tumours were not found in nude mice injected with NIH 3T3 cells, 3T3-pMV7 or 3T3-pMV7-4E (Ala). Tumours showed unlimited growth, and were categorized upon dissection as malignant fibrosarcomas (S. Jothy, A.L.-K. and N.S., data not shown).

To confirm that eIF-4E possessed transforming activity, we have repeated the experiments with Rat 2 cells, another

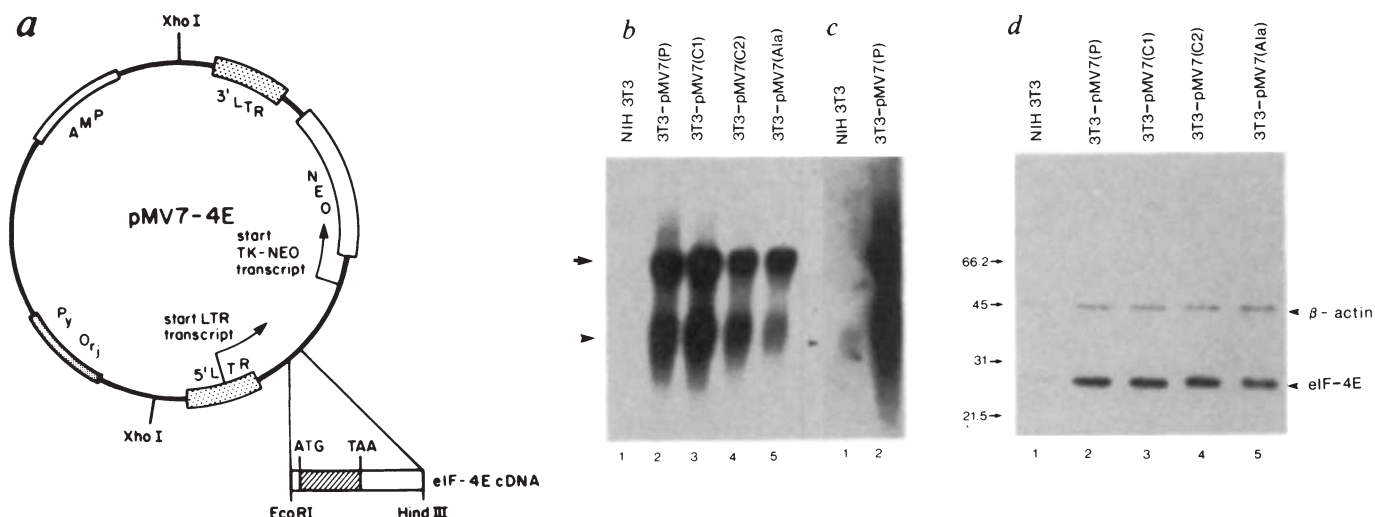


FIG. 1 Overexpression of eIF-4E. *a*, Schematic diagram of the pMV7 expression system. Murine eIF-4E cDNA (ref. 29; a 1.6-kb clone containing 20 bp 5' untranslated region (UTR), 654 bp coding sequence and 956 bp of 3' UTR, was digested with *EcoRI*, which preserves the 5' UTR, and *HindIII*, downstream of a polyadenylation signal, to generate a 1.4 kb fragment) was subcloned into the retroviral expression vector pMV7 (ref. 17), downstream of the Moloney MuLV (Mo-MuLV) long terminal repeat (LTR). The herpes simplex virus thymidine kinase (TK) promoter drives expression of the bacterial neomycin-resistance gene (neomycin phosphotransferase). pMV7-4E (Ala) contains a mutation of ACG to GCC, created by site-directed mutagenesis in M13 (ref. 30), which changes a serine to alanine at position 53 of the protein. The mutated clone was sequenced in its entirety to ensure that other mutations have not occurred during mutagenesis. NIH 3T3 fibroblasts were transfected and grown as previously described³¹. Briefly, ψ 2 cells were electroporated with $30 \mu\text{g ml}^{-1}$ G418 (neomycin) for 10 days. The virus was collected and used to infect 1×10^6 NIH 3T3 cells in 100-mm dishes. G418-resistant colonies were isolated after 10 days of selection. The remaining colonies were pooled and grown into mass culture and referred to as (P). A control cell line was derived from infection of NIH 3T3 cells with virus containing the pMV7 vector. *b*, Northern blot analysis of eIF-4E mRNA. Total cellular RNA was isolated by the guanidium-thiocyanate method and enriched for poly(A)⁺-containing RNA by two cycles of oligo (dT)-cellulose chromatography by standard procedures³². Poly(A)⁺ RNA (2 μg) was subjected to electrophoresis through a 1.25% agarose gel containing 6% formal-

dehyde in $1 \times \text{MOPS}$ (morpholinopropanesulphonic acid) buffer. RNA was transferred to a Gene-screen-plus membrane and hybridized to [α -³²P]dATP random-primed probe, containing the entire coding region of the eIF-4E cDNA. Prehybridization and hybridization was carried out at 65 °C using 10^6 c.p.m. ml^{-1} of probe. Filters were washed twice in $1 \times \text{SSC}/0.1\%$ SDS at 60 °C followed by $0.5 \times \text{SSC}/0.1\%$ SDS for 60 min at 65 °C. The membrane was dried and exposed against X-ray film for 6 h. *c*, A longer exposure (4 days) of lanes 1 and 2 of panel *b*. *d*, Immunoblot (western) analysis. Cells were washed three times with ice-cold PBS (140 mM NaCl, 15 mM KH_2PO_4 pH 7.2, and 2.7 mM KCl) and incubated on ice with 1 ml buffer containing 0.5% Nonidet P-40 (NP40), 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 50 mM NaF and 2 mM PMSF for 15 min to achieve lysis. The lysate was centrifuged at 10,000g for 20 min and an aliquot containing an equal amount of protein (determined by Bradford assay) was electrophoresed on a 12.5% SDS-polyacrylamide gel followed by transfer to nitrocellulose membrane in 25 mM Tris-HCl (pH 7.5), 190 mM glycine and 40% methanol for 1 h at 1 amp. The filters were blocked with 5% non-fat dried skim milk powder in PBS for 1 h at room temperature, and incubated with both a polyclonal rabbit antibody against a mouse eIF-4E synthetic peptide (amino acids 5–23; EPETTPPTNPPPAEEEEKT (single-letter code) and a monoclonal mouse β -actin antibody (NEN) for 16 h. After extensive washings in PBS, the filters were incubated for 2 h with $0.1 \mu\text{Ci ml}^{-1}$ of both ¹²⁵I-protein A to detect eIF-4E, and ¹²⁵I-goat anti-mouse antiserum (NEN) to detect β -actin, washed, dried and exposed to X-ray film. Relative molecular masses are shown as $M_r \times 10^{-3}$.

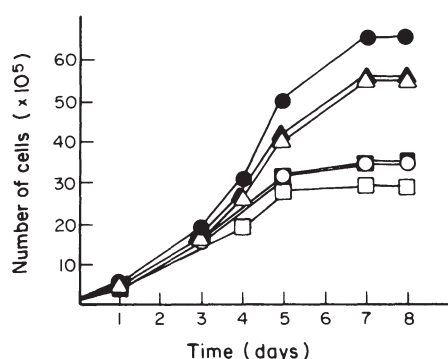


FIG. 2 Growth curves of control cell lines and cell lines overexpressing eIF-4E. Cells were seeded at 1×10^5 cells per 35-mm plate and grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, in a 37 °C, 5% CO₂ humidified environment. Cells were counted on the indicated days with a Coulter counter. All results are the mean of three experiments carried out in duplicate. ●, 3T3-pMV7-4E(P); △, ▲, 3T3-pMV7-4E (C1 & C2); ■, NIH 3T3; ○, 3T3-pMV7; □, 3T3-pMV7-4E (Ala).

established non-transformed cell line. As with NIH 3T3, overexpression of eIF-4E in Rat 2 cells resulted in transformation *in vitro* (data not shown). Injection into nude mice of the cells overexpressing eIF-4E induced tumour formation (data not shown). Rat 2 control cells and cells infected with pMV7 were not transformed.

The tumorigenic capacity of eIF-4E agrees with many other observations linking eIF-4E to growth control circuitries. Activation of eIF-4E through phosphorylation occurs after stimulation by diverse extracellular agents including serum, platelet-derived growth factor (R. Frederickson and N.S., unpublished results), insulin (J. Traugh, personal communication), TNF (ref. 15), and TPA (ref. 16). Significantly, viral tyrosine-kinase oncoproteins, such as *v-src* or *v-fps*, also enhance phosphorylation of eIF-4E (R. Frederickson and N. S., unpublished results). Another strong indication for the involvement of eIF-4E in control of cell growth is derived from genetic studies in yeast. The cell-cycle mutant of *S. cerevisiae*, CDC-33, harbours a mutation in eIF-4E (refs 22, 23). This mutation can be complemented by a mutation in the regulatory subunit of cyclic AMP-dependent kinase, suggesting that phosphorylation of eIF-4E has a crucial role in regulating its activity²². These results are consistent with a central role of eIF-4E phosphorylation during development. For example, after fertilization, eIF-4E phosphorylation in sea urchin oocytes increases, and subsequently decreases during mitosis (A. Lopo, personal communication).

The mechanism by which eIF-4E transforms cells is not clear. The expression of certain oncogenes or growth factors might be tightly controlled at the translational level by the availability of

eIF-4E. Increasing the concentration of eIF-4E would thus relieve translational repression. In this regard, it is interesting that several proto-oncogenes seem to be translationally regulated either *in vivo* or *in vitro*. These include *c-sis* (ref. 24), *lck* (ref. 25) and *c-myc* (ref. 26). Significantly, the mRNA 5' non-coding region of these and many other proto-oncogenes are different from the majority of cellular mRNAs in that they contain a long 5' non-coding region. Translation of many of these mRNAs is inefficient in reticulocyte lysates *in vitro*, but is enhanced by the truncation of the long 5' non-coding region^{27,28}. Experiments *in vivo* with *c-sis* and *lck* led to similar conclusions^{24,25}. We propose, on the basis of the results in this paper, that a selective mechanism exists for increasing the translational efficiency of inefficiently initiated mRNAs such as oncogene mRNAs. This mechanism implicates a nonspecific enhancement of the efficiency of translational initiation by overexpression of eIF-4E.

We suggest that eIF-4E is an intracellular transducer of extracellular signals and an intermediary in growth control pathways. eIF-4E is the substrate for a kinase(s) in the phosphorylation cascade triggered by diverse mitogenic agents and tyrosine kinases (discussed above). One intriguing possibility is that eIF-4E is a necessary, but not sufficient, mediator of the mitogenic response by tyrosine kinases. Thus, tyrosine kinases might induce eIF-4E phosphorylation through one or more intermediates, thereby activating eIF-4E for the enhanced translational expression of oncogenes. Consistent with our hypothesis are recent findings that injection of wild-type recombinant eIF-4E (expressed in *Escherichia coli*) into quiescent NIH 3T3 cells

TABLE 1 Growth properties of cell lines overexpressing eIF-4E and of controls

Cell line	Growth in monolayer cells		Growth in agar		Tumorigenicity	
	Doubling time (h ± s.d.)	Saturation density ($\times 10^6 \pm$ s.d.)	Efficiency (%)	Colony size (mm)	Tumours/ injection	Latency (days)
NIH 3T3	27 ± 1.1	3.4 ± 0.2	0	—	0/5	—
3T3-pMV7	27 ± 1.0	3.2 ± 0.1	0	—	0/5	—
3T3-pMV7-4E(P)	20 ± 1.4	6.4 ± 0.7	20	0.6–1	8/8	10–12
3T3-pMV7-4E(C1)	23 ± 1.4	5.6 ± 0.3	19	0.5–0.8	6/6	14–15
3T3-pMV7-4E(C2)	22 ± 1.2	5.7 ± 0.8	18	0.5–1	7/7	12–15
3T3-pMV7-4E(Ala)	31 ± 1.0	2.8 ± 0.3	0	—	0/4	—

The data presented for 3T3-pMV7 and 3T3-pMV7-4E (Ala) cells represents polyclonal populations. Doubling time and saturation densities were measured by seeding $\sim 1 \times 10^5$ cells per 35-mm cell culture well in DMEM supplemented with 10% FCS. Cells were trypsinized and counted after 12 h and fed every 2 days. Doubling time was calculated from the growth curves shown in Fig. 2. Saturation densities were determined by allowing the cells to grow for 4 days after reaching confluency. All results are the mean of three experiments performed in duplicate. For plating in soft agar, $\sim 5 \times 10^4$ cells were resuspended in 2 ml of 0.35% (wt/vol.) agar solution, containing DMEM plus 20% FCS, overlaid onto a 0.5% (wt/vol.) agar solution in a 35-mm plate. Two days after incubation, 2 ml of DMEM supplemented with 20% FCS was added. Colonies were counted 16–21 days after plating. Cloning efficiency in agar was calculated by the number of colonies $\times 100$ divided by the number of cells plated. Colonies were scored as cell aggregates containing more than 10 cells. To test for tumorigenicity 4–8-week-old athymic nude mice (CD1 nu/nu; Charles River) were injected subcutaneously on the left side near the lower limb, with 2.5×10^6 cells resuspended in 100 µl PBS. Mice that developed tumours were killed after 21 days, at which time the average diameter of the tumour was 2.5–3.0 cm. Mice that did not develop tumours were observed for 70 days.

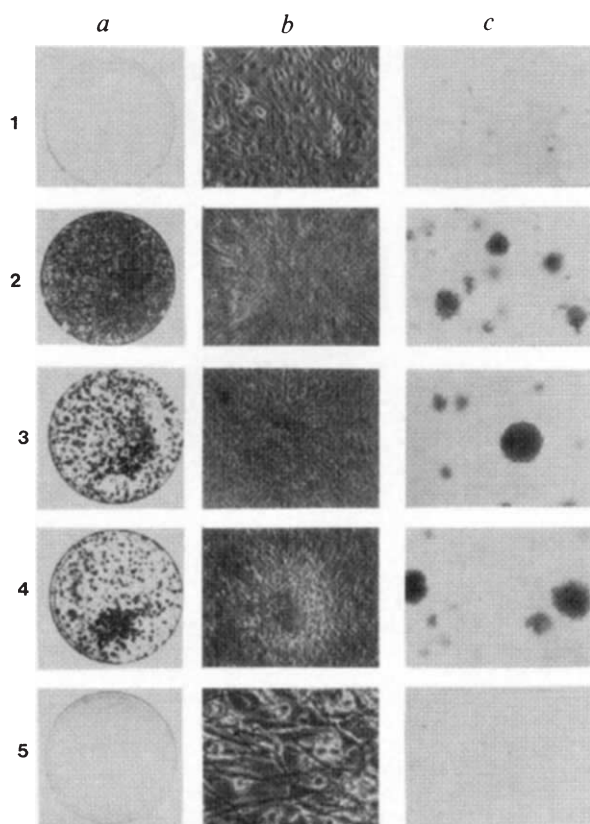


FIG. 3 Characteristics of cell lines overexpressing eIF-4E in monolayers and soft agar. *a* and *b*, Growth in monolayers. Control NIH 3T3(1), 3T3-pMV7-4E(P)(2), (C1)(3), (C2)(4) and (Ala)(5) cell lines were plated at 1×10^5 cells and grown to confluency in 100-mm dishes and maintained for 7 days in DMEM plus 10% FCS, with the addition of fresh medium every 2 days and photographed. *a*, Cells stained with methylene blue. Cells were fixed in 10% formaldehyde for 2–3 h, rinsed and stained with 0.1% methylene blue. The number of foci observed for 3T3-pMV7-4E(P), 3T3-pMV7-4E(C1) and 3T3-pMV7-4E(C2) are >1000, 700 and 600, respectively. Approximately 75% of the primary G418-resistant colonies appeared morphologically transformed. *b*, $\times 160$ magnification. *c*, Anchorage-independent growth. The procedure for growth in soft agar was as described in Table 1. $\times 160$ magnification of colonies in soft agar.

induced DNA synthesis (M. Smith, M. Jaramillo, H-F. Kung and N.S., submitted).

The finding that a translation factor is a proto-oncogene indicates that regulation of gene expression at the translational level is important for cell growth. Elucidating the mechanism by which excess amounts of eIF-4E transform cells would contribute significantly to our knowledge of the control of cell growth during differentiation, development and neoplasia. □

Received 22 December 1989; accepted 22 March 1990.

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ACKNOWLEDGEMENTS. We thank Vinita Adkar for technical assistance, Drs John Bell, Serge Jothy and Andre Veillette for advice and Drs P. Gros, C. Stanners, M. Szyf, A. Veillette, and members of our laboratory for comments on the manuscript. We thank Drs R. Krauss and B. Weinstein for plasmid pMV7. This work was supported by the National Cancer Institute of Canada and the Medical Research Council of Canada.

Identification of a functional intermediate in receptor activation in progesterone-dependent cell-free transcription

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THE steroid hormone, progesterone, enhances transcription *in vivo* from promoters containing progesterone response elements (PREs)^{1–5}. We have recently shown that the progesterone receptor (PR) modulates transcription in a cell-free system by facilitating the formation of a stable preinitiation complex, apparently through interaction with RNA polymerase II and other basic transcription factors^{6,7}. The precise role of ligand in this activation process remains unclear, however. In order to dissect the role of steroid ligand in gene action, we sought to devise an *in vitro* transcription system that mimics the hormone-dependent transcriptional activation observed *in vivo*. We now report the successful reconstitution *in vitro* of progesterone-dependent RNA synthesis from a PRE-driven promoter in nuclear extracts of human breast carcinoma (T47D) cells. The transcriptional activation is triggered by the hormone-induced binding of endogenous PR to PREs and exhibits hormone-specificity. The receptor exists in a 4S form in our initial salt-treated extract and is apparently dissociated from the heat-shock protein hsp90. Nevertheless, hormone is still required for DNA binding and transcriptional activation. These results suggest that dissociation of hsp90 and conversion to an inactive 4S intermediate could occur before the final event in ligand-mediated transactivation of gene expression.

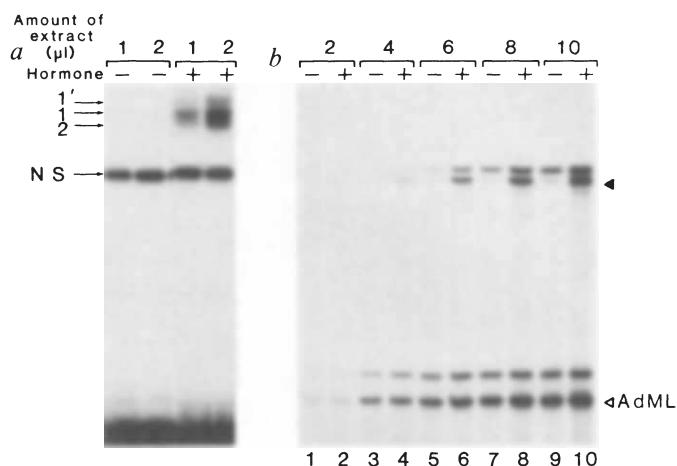
T47D cells are enriched in PR and considerable amounts of receptor are present in the crude nuclear fraction even in the absence of hormone. We tested nuclear extracts of T47D cells, grown in hormone-deprived conditions, for DNA binding and transcriptional activity in the presence or absence of added hormone. Figure 1a shows that in a gel retardation experiment the hormone-free receptors did not generate any specific DNA-protein complex with PRE. Incubation of the extract with hormone induced the formation of specific DNA-protein complexes, however. In a previous study using monospecific antibodies against PR, we have established that these complexes were indeed formed by binding of A and B forms of PR to PRE

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Fig. 1 Progesterone induces DNA binding and transcriptional activation *in vitro*. *a*, Gel retardation experiments were carried out as described previously^{5,8} with hormone-free or hormone-treated T47D nuclear extracts. A double-stranded oligonucleotide of 23 base pairs (bp) containing the PRE from the tyrosine amino transferase (TAT) gene³ was end-labelled and used as probe. Nuclear extracts were preincubated at 25 °C with or without 10^{-7} M progesterone for 15 min. Aliquots (1 and 2 μ l) of the treated extracts were then combined with 0.1 ng labelled PRE and 1 μ g poly(dI-dC), incubated at 25 °C for 15 min and analysed by gel retardation assay. The arrows marked 1, 1' and 2 indicate the DNA-protein complexes formed by hPR. NS indicates a non-specific DNA-protein complex. *b*, Nuclear extracts were treated with or without hormone as described in *a*. Construction of DNA templates and conditions for *in vitro* transcription assay were as described previously⁶. The test template was constructed by placing two copies of PRE 20 bp upstream of a sequence containing the ovalbumin TATA box and linked to a 377-nucleotide G-free cassette⁶. Correct initiation of transcription from this promoter results in the synthesis of a 360-nucleotide transcript. Random initiation upstream of the G-free cassette would generate longer transcripts which would then be processed by T1 RNase to a particular length of 378 nucleotides. We used a second promoter containing adenovirus major late (AdML) sequences (-400 to +10) and linked to a 200-nucleotide G-free cassette as an internal promoter control. The amount of transcript generated was measured by estimating the 32 P counts present in individual bands in the dried gel in a Betagen analyser. The increased stimulation of hormone-induced correct initiation was calculated after normalization relative to AdML control. A typical transcription assay mixture contained 100 ng (30 fmol) of test PRE₂TATA template, 50 ng (15 fmol) of control AdML template, 2–10 μ l (10–50 μ g) of T47D nuclear extract treated with or without hormone and other transcription reaction components. The nuclear extracts were preincubated with template DNA at 25 °C for 10 min before the addition of the transcription mixture. The final NaCl concentration in the reaction was maintained at 60 mM.

METHODS. T47D human breast cancer cells were cultured as described previously²¹ and switched to a growth medium containing 5% charcoal-stripped fetal calf serum at least 5 days before collection. Near-confluent flasks of cultured cells ($\sim 20 \times 10^6$ – 30×10^6 cells per flask) were collected using Hank's balanced salt solution containing 1 mM EDTA. The cell pellet (≈ 3 ml) was then washed with 30 ml ice-cold PBS. All subsequent steps were carried out at 4 °C. The washed pellet was resuspended in 15 ml buffer A containing 15 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% glycerol and 2 mM DTT and spun for 10 min at 5,000 r.p.m. in a JA-20 Beckman rotor. The supernatant was discarded and the pellet resuspended in 6 ml buffer A.

sequences⁸. To study the effects of hormone on the transcriptional activities of these extracts, we used a synthetic promoter containing two copies of a PRE and the ovalbumin TATA box as described in the legend of Fig. 1. Treatment of nuclear extracts with 10^{-7} M progesterone resulted in a 8–10-fold induction in the synthesis of the correctly initiated (360 nucleotides) transcript (Fig. 1*b*). Very little accurately initiated transcription occurred in the absence of hormone. This hormonal induction of transcription was observed over a wide range of concentrations of nuclear extract. Hormone-induced transcriptional enhancement was entirely promoter-specific as the activity of the adenovirus promoter remained unchanged on hormone treatment. These results showed clearly that treatment of PR with progesterone alone results in the activation of RNA syn-



Cells were lysed by 20 strokes of a B-pestle in a dounce homogenizer. The homogenate was centrifuged at 5,000 r.p.m. for 10 min, and the supernatant was removed. The crude nuclear pellet was resuspended in 2 ml of buffer C containing 20 mM HEPES pH 7.9, 20% glycerol, 0.6 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 2 mM DTT and was homogenized again. The homogenate was transferred to a 30-ml plastic centrifuge tube and incubated on ice for 30 min with resuspension every 5 min. The suspension was then spun at 15,000 r.p.m. for 30 min. The supernatant (nuclear extract) was collected and dialysed against 100 volumes of buffer D containing 20 mM HEPES pH 7.9, 20% glycerol, 100 mM NaCl, 0.2 mM EDTA, 5 mM MgCl₂ and 2 mM DTT for 4 h with one change of buffer. The dialysed extract was centrifuged at 15,000 r.p.m. for 20 min and frozen in small aliquots. Protein concentration was typically 3–5 mg ml⁻¹. Receptor concentration was measured by steroid-binding assay. Aliquots (50 μ l) were incubated with 20 mM [³H]R5020 for 4 h at 4 °C in the presence or absence of unlabelled R5020. Unbound hormone was removed by charcoal stripping and the supernatant was counted for radioactivity. PR concentration was about 10–12 pmol ml⁻¹. By our estimates, the number of receptor molecules (100–120 fmol) is about twofold higher than the number of PRE elements (60 fmol) in the transcription assay. Each PRE can, however, bind two receptor molecules⁵.

thesis from a PRE-driven promoter and that this was concurrent with hormonal induction of high-affinity binding of PR to PREs.

To test the PRE-dependence of the hormone-induced transcriptional enhancement, we used a promoter which exactly resembled the test promoter except for a lack of PRE sequences. Progesterone failed to induce the synthesis of any correctly initiated transcripts from a promoter that did not contain PREs whereas it stimulated transcription from the PRE-containing promoter (Fig. 2*a*). We investigated the PRE requirement for transactivation by carrying out competition experiments with oligonucleotides containing the PRE sequence (Fig. 2*b*). A 20-fold molar excess of the PRE oligonucleotide efficiently inhibited accurate transcription from the test promoter whereas transcription from the control adenovirus major late promoter remained

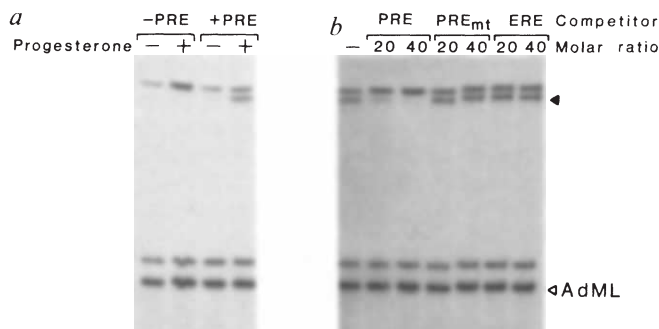


FIG. 2 Progesterone-induced transcription *in vitro* is PRE-dependent. *a*, Transcription *in vitro* was carried out with extracts (10 μ l) with or without 10^{-7} M progesterone. The PRE-less promoter pLOVTATA was identical to the template PRE₂ TATA but lacked the PREs⁸. Lanes indicated by -PRE and +PRE represent reactions containing the PRE-less and PRE-containing templates. *b*, Synthetic oligonucleotide duplexes containing TAT gene PRE³, a mutant PRE³ and ERE (oestrogen response element)²² were mixed with the test template before addition of the hormone-treated nuclear extract. The molar ratios of the competitor oligonucleotides to the test template are indicated.

unaffected. An oligonucleotide harbouring oestrogen-responsive element (ERE) sequences⁹ or a mutant PRE oligonucleotide carrying two point mutations, which drastically reduce its binding to PR and impair its function *in vivo*^{3,5}, failed to prevent accurate RNA synthesis from the authentic test promoter. Our results show that the hormone-induced transcriptional stimulation is mediated by a transactivator that binds to PRE sequence with specificity and high affinity, clearly indicating that native PR mediates the progesterone-induced transcriptional response.

We next examined the hormonal specificity of the transcriptional enhancement. The specific DNA-binding capacity of PR in T47D extracts was induced by progesterone only and not by oestrogen (Fig. 3a, left panel). Consistent with the DNA-binding results, progesterone, but not 17 β -oestradiol nor cortisol, enhanced transcription *in vitro*. A dose-response experiment with progesterone indicated that maximum transcriptional stimulation was achieved at a progesterone concentration of

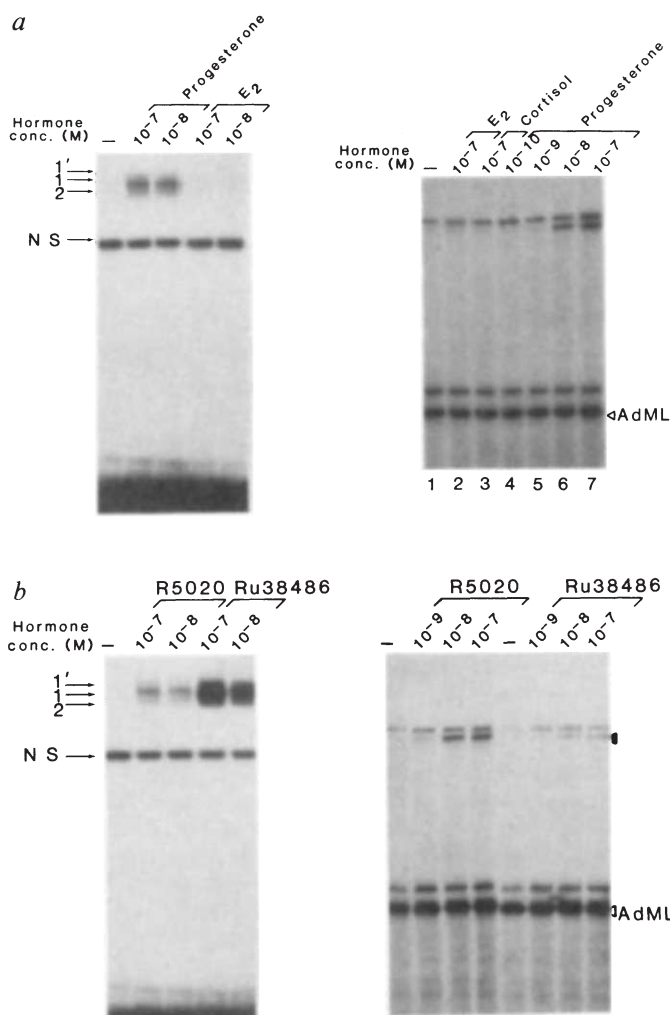


FIG. 3 *a*, Specificity of hormonal activation of DNA binding and transcriptional enhancement by PR. Left panel, Nuclear extracts were treated with or without indicated concentrations of progesterone, 17 β -oestradiol or cortisol and 1- μ l samples were assayed by gel retardation as described in Fig. 1. Right panel, nuclear extracts were treated with various hormones as indicated and 10 μ l of each extract was assayed for transcriptional activity *in vitro*. *b*, Effects of progestin R5020 and antiprogestin Ru-486 on DNA binding and transcription *in vitro*. Left panel, DNA-binding assays were performed by gel retardation with nuclear extracts treated with or without R5020 or Ru-486. Right panel, transcription *in vitro* was carried out with the extracts treated as described above.

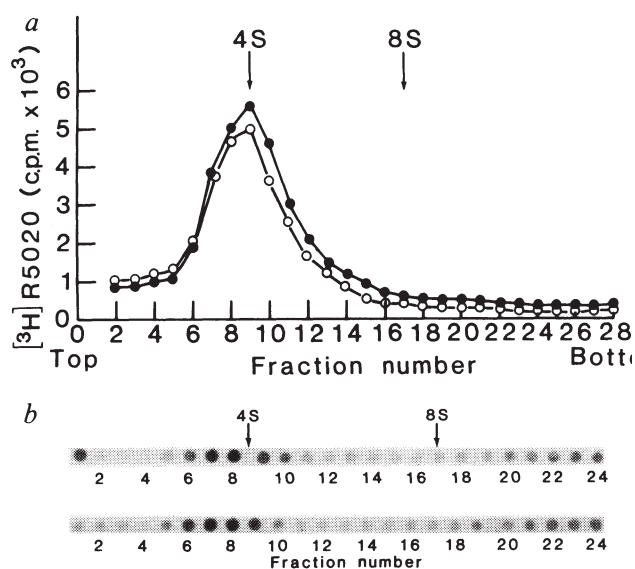


FIG. 4 Progesterone receptor exists in 4S form in nuclear extracts of T47D cells. *a*, Nuclear extracts were labelled with 10⁻⁷ M [³H]R5020 at 4 °C for 1 h in the presence or absence of 20 mM sodium molybdate. Unbound steroid was removed by adsorption with dextran-coated charcoal. The samples were then loaded on 0–30% sucrose gradients containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 12 mM 1-thioglycerol, 5% glycerol, 20 mM molybdate and 150 mM NaCl. The gradients were spun in a vertical rotor at 4 °C for 90 min and then fractions were collected. Each fraction was counted for radioactivity. Control tubes containing chick oviduct cytosol stabilized by molybdate and labelled with tritiated hormone in the presence or absence of molybdate respectively. The positions of migration of 4S and 8S complexes in the gradient are marked by arrows. *b*, Nuclear extracts with or without hormone treatment were subjected to ultracentrifugation in molybdate containing sucrose gradients as described above. The collected fractions were blotted on nitrocellulose and probed with a monoclonal antibody AB52 against human PR followed by a rabbit anti-mouse antibody and finally ¹²⁵I-labelled protein A. The upper and lower rows of dots represent extracts treated with or without hormone respectively.

10–100 nM (Fig. 3a, right panel). These results correlate well with the physiological parameters of PR in T47D cells and substantiate the involvement of PR itself in mediating hormonal induction of transcription.

The compound Ru38486 has strong anti-progestin and anti-glucocorticoid activity *in vivo*^{10–14}. The molecular basis of these activities is not understood. Our experiments showed that human PR complexed with Ru38486 had a PRE-binding capacity even greater than that of PR bound to progestin R5020 (Fig. 3b, left panel). The increased binding to PREs by Ru38486-PR complex might be due to an altered structure which causes it to bind to PREs with higher affinity and stability^{8,15}. Consequently, we tested the transcriptional activity of Ru38486-PR in our *in vitro* system and compared it with that of R5020-PR. Treatment of PR with 10–100 nM Ru38486 also led to transcriptional activation of the test promoter (Fig. 3b, right panel). The extent of this transcriptional enhancement, however, was only about 25–30% (an average of three experiments) of that elicited by the progestin agonist R5020. The much weaker transcriptional activation by the Ru38486-PR complex may be the result of an altered receptor conformation which affects its interaction with the transcriptional machinery. Nevertheless, the fact that Ru38486 treatment can induce even a weak transcriptional activation indicates that at least part of the antagonistic action shown by Ru38486 *in vivo* could be exerted at an earlier step, before DNA binding (the 8S to 4S transformation step for example). It is quite possible that in our *in vitro* experimental protocol we have by-passed this earlier inhibitory step.

To ascertain the structural status of the PR in the salt extracts of nuclei of hormone-free T47D cells, we analysed the sedimentation behaviour of this unfractionated PR in various conditions (Fig. 4). The crude receptor preparation was labelled with tritiated R5020 in either the presence or the absence of sodium molybdate and subjected to rapid ultracentrifugation in sucrose gradients. As one would expect, in the absence of molybdate the R5020-PR complex migrated in a single 4S peak. Even in the presence of molybdate, which stabilizes an 8S form of receptor¹⁶, the R5020-PR complex sedimented in a 4S form (Fig. 4a). In a control experiment, the molybdate-stabilized chick PR labelled with R5020 migrated as an 8S complex (data not shown). These results suggested that in our nuclear salt extract, the 8S form of receptor had already undergone transformation to a 4S form.

To confirm these results, the molybdate-stabilized nuclear extract was analysed by sucrose gradient ultracentrifugation in the absence of hormone, and various gradient fractions were probed by immunoblotting with a monospecific antibody AB52 (ref. 17) (Fig. 4b). The results of this experiment indicated again that in a hormone-free state, the receptor existed in a 4S form after salt extraction of the nuclei. Our results are consistent with the results of numerous laboratories who have reported that the high-salt mediated dissociation of hsp90 from receptors leads to a conversion from an 8S to a 4S structural complex^{12,16,18,19}.

The observation that a 4S form of PR requires hormone to be transcriptionally active provides a novel and interesting insight into the catalytic role of hormone in receptor-mediated gene activation. Although the 4S PR is apparently free of hsp90, it remains transcriptionally inactive. The receptor may, however, still be associated with other lower molecular mass inhibitory proteins which mask its dimerization, DNA binding and/or transactivation properties. In contrast to hsp90, hsp70 remains associated with glucocorticoid and progesterone receptors even in its 4S form²⁰. In such a scenario, the role of the hormonal ligand could be to bind to the receptor, induce a structural change and trigger the release of some inhibitory protein(s). The free receptor could then acquire the proper conformation to bind to DNA and effect gene transactivation. We have reported previously that highly purified chicken PR can bind to target DNA and enhance transcription in a hormone-independent manner⁶. It is conceivable that during purification, various *in vitro* manipulations may have accomplished an irreversible conformational change in the purified receptor and converted it to a form which can effect transactivation without ligand binding. *In vivo* and in crude extracts, the receptor may not have undergone such a conformational change and hormone would be absolutely necessary to transform it into a form that is active in DNA binding and transactivation.

Our results are consistent with a two-step model for ligand-induced activation of the receptor. Before addition of hormone, PR is present in the cell in an inactive 8S complex with hsp90 and other inhibitory proteins^{21–24}. Hormone treatment results in the release of hsp90 and the conversion of the 8S to a 4S form²⁵. Our experimental set-up has by-passed this hormone-induced transformation event as salt treatment *in vitro* has converted the 8S to the 4S form. This cell-free system has allowed us to trap a previously unidentified intermediate form of the receptor—a 4S form which is not competent to bind specifically to DNA and induce initiation of transcription. As dissociation of hsp90 and conversion to 4S is not sufficient to make the receptor competent for transactivation, we propose that an additional conformational alteration(s) in the receptor molecule must precede the activation of target genes by PR. □

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ACKNOWLEDGEMENTS. We thank Dr Dean P. Edwards for providing the monoclonal antibody AB52. We also thank M. L. Wolfe for technical help and L. Gamble for preparation of this manuscript. This work was supported by the NIH.

C-terminal truncated glucose transporter is locked into an inward-facing form without transport activity

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THE facilitated glucose transporters comprise a structurally related family of proteins predicted to have 12 membrane-spanning domains, with the amino terminus, a relatively large middle loop and the carboxy-terminus all oriented towards the cytoplasm^{1–10}. An alternating conformation model has been proposed to explain the mechanism of facilitated glucose transport^{11–17}. To understand the structure–function relationships, especially the role of the intracellular C-terminal domain, we have modified the rabbit equivalent of the erythroid-type transporter, GLUT1 (ref. 18), using complementary DNA to code for a deletion mutant that lacks most (37 out of 42 amino acids) of the intracellular C-terminal domain. This deletion mutant is expressed at the cell surface of Chinese hamster ovary (CHO) cells, but is functionally inactive, probably because it has lost its capacity to alternate in conformation and so is locked into an inward-facing form.

Successful expression of the deletion mutant is shown in Fig. 1a and b. Western blotting of a stable CHO transfectant cell line (designated clone DC37) with an anti-peptide antibody directed against residues 215–226 of the glucose transporter¹⁹ specifically identified the truncated glucose transporter protein (relative molecular mass (M_r) 46,000 (46K)) (Fig. 1a, lane 3). In contrast, CHO cells expressing the normal (wild-type) rabbit GLUT1 protein, which was previously designated clone B (ref. 20), had a similar amount of expressed 50K glucose transporter protein (lane 4). The apparent differences in the transporter molecular weight between clone DC37 and clone B directly correspond to the deletion of 37 amino acids (~4K). The deletion of the C-terminal domain of the glucose transporter in clone DC37 was confirmed using an anti-peptide antibody directed

Received 18 January; accepted 26 March 1990.

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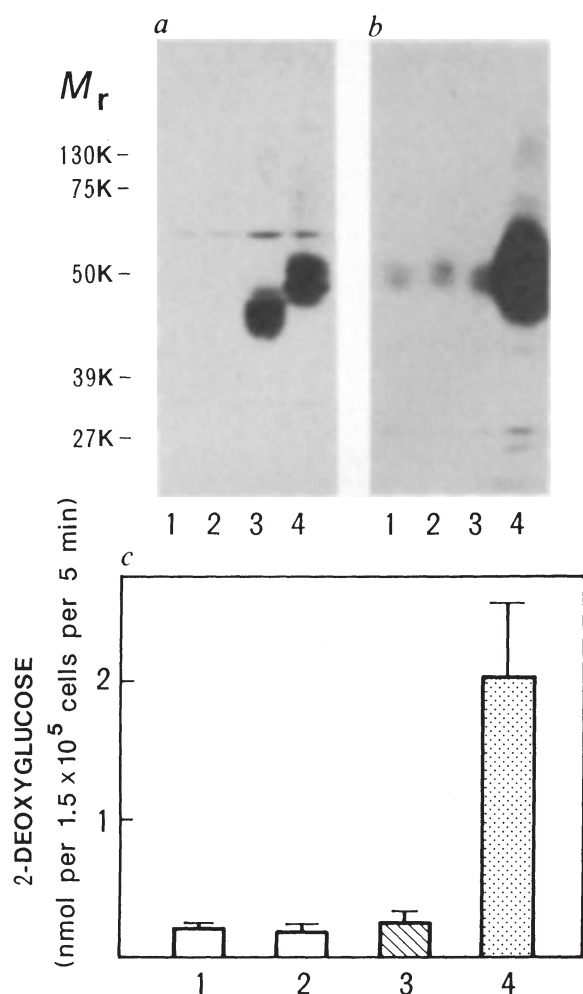
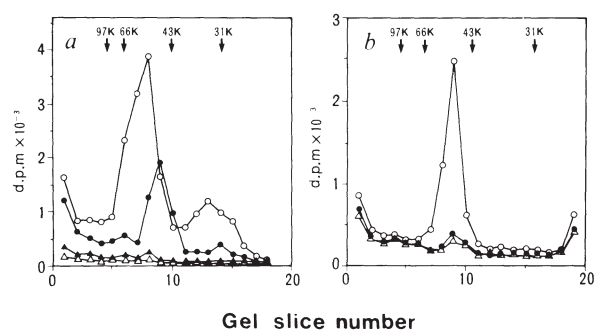


FIG. 1 Expression of the C-terminal deletion mutant of rabbit GLUT1 glucose transporter protein and its effect on transport activity in CHO cells. Western blotting was performed with antisera raised against a synthetic peptide corresponding to residues 215–226 (a) or residues 478–492 (b) of the glucose transporter. Membranes were prepared from control CHO cells (lane 1), CHO cells transfected with expression vector alone (lane 2), CHO cells with expressed C-terminal truncated mutant of glucose transporter, designated clone DC37 (lane 3), and CHO cells with expressed normal rabbit GLUT1 glucose transporter, termed clone B (ref. 20) (lane 4). 2-Deoxy-D-glucose uptake (c) was determined in control CHO (bar 1), vector alone (bar 2), clone DC37 (bar 3) and clone B (bar 4) corresponding to the same cell lines used for membrane preparations in a and b.

METHODS. The *Mae*I fragment of the rabbit GLUT1 glucose transporter cDNA (nucleotide 111–2,230)³ was digested with exonuclease III and ligated in the presence of a universal translation terminator (Pharmacia). Sequencing of this deleted cDNA revealed that the translated protein was expected to have residues 1–454 of the rabbit GLUT1 glucose transporter with the C-terminal Ala 455 replaced with Thr. This mutated DNA was ligated into expression vector pMTNeo containing the mouse metallothionein I promoter and the G418 (a neomycin derivative) resistance gene as previously described²⁰ and transfected into CHO cells by calcium phosphate precipitation. The cells were selected in the presence of $600 \mu\text{g ml}^{-1}$ of G418, and one clone, DC37, was selected for these studies. Cells were homogenized in 10 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 250 mM sucrose, pH 7.4, in Potter–Elvehjem glass–Teflon type homogenizer at 4 °C. The homogenates were centrifuged at 900g for 10 min to sediment the fraction containing mainly nuclei and the resulting supernatant was centrifuged at 17,000g for 75 min at 4 °C. The pellet (100 μg) was subjected to SDS-polyacrylamide (10%) gel electrophoresis and transferred onto a nitrocellulose filter. Filters were incubated with antisera raised against a synthetic peptide corresponding to residues 215–226 (a) or residues 478–492 (b) at a 1:20 dilution. The production and characterization of these antibodies have been described previously¹⁹. Filters were then incubated with ^{125}I -protein A (Amersham) and autoradiographed. c, 2-Deoxy-D-glucose uptake in CHO cells was measured as described²⁰. Near-confluent monolayers of CHO cells in 24-well plates were incubated with 0.1 mM 2-deoxy-D-[1,2- ^3H]glucose (ICN) for 5 min at 37 °C. The reaction was stopped by the addition of ice-cold PBS (10 mM) with 0.3 mM phloretin. Cells were washed and solubilized and radioactivity was measured. Data are the mean $\pm$ s.d. of 4 separate experiments.

FIG. 2 Cell-surface labelling of the glucose transporter with galactose oxidase and borotritide (a) and photoaffinity labelling with ATB-BMPA (b). a, Surface glycoproteins were labelled with galactose oxidase and sodium borotritide in intact cells and subsequently immunoprecipitated with antibodies against the glucose transporter peptide. The glucose transporter in clone B was immunoprecipitated with control IgG (Δ) or with an antibody against the C-terminal domain ($\circ$). The C-terminal truncated glucose transporter in clone DC37 was immunoprecipitated with an antibody against the C-terminal domain ($\blacktriangle$) or against the middle cytoplasmic domain ($\bullet$). b, Photoaffinity labelling of glucose transporter with ATB-BMPA in intact control CHO cells (Δ), clone DC37 ($\bullet$) and clone B ($\circ$).

METHODS. a, Cell-surface glycoproteins were labelled as described²¹, with some modifications. The CHO cells in the culture dish (diameter 60 mm) were incubated with 1 unit ml^{-1} neuraminidase at 22 °C in PBS (20 mM), pH 6.0, for 15 min. Labelling was at 15 °C. Cells were then pre-reduced by addition of 8 μl 250 mM sodium borohydride in 100 mM NaOH to 1 ml of PBS (20 mM), pH 8.0. After 5 min, cells were washed and incubated with 40 units of galactose oxidase in 1 ml PBS (20 mM), pH 7.4, for 30 min, then reduced by the addition of sodium borotritide (1 mCi) to 1 ml of PBS (20 mM), pH 8.0. After 10 min, 1 ml PBS (20 mM), pH 7.4, was added and the cells were incubated for a further 5 min. Cells were then washed and solubilized with 2% dodecyl octaethyleneglycol ether (C_{12}E_8) in 50 mM HEPES, 150 mM NaCl, pH 7.6, containing 1 mM PMSF. The cell lysate was centrifuged at 13,000g for 30 min at 4 °C. The labelled glucose transporter in the supernatant was immunoprecipitated first with 30 μg control rabbit IgG and 25 μl protein A-Cellulofine (Seikagaku Kogyo, Japan), and the remaining supernatant was then immunoprecipitated with 30 μg anti-peptide antibody against the C-terminal domain (residue 478–492) and 25 μl of protein A-Cellulofine. For clone DC37, the resulting supernatant was again immunoprecipitated with 30 μg anti-peptide antibody directed against the



middle cytoplasmic loop of the glucose transporter (residues 215–226) and protein A-Cellulofine. The immunoprecipitates were suspended with 2% SDS (lauryl grade, Pierce), 20 mM DTT and 6M urea and then subjected to SDS-PAGE (10%). Gel lanes were sliced and the incorporated tritium in each gel slice (5 mm long) was determined¹⁹. Results with control IgG from clone DC37 were the same as those with control IgG from clone B and have been omitted for the sake of clarity. Experiments were carried out three times with similar results. b, CHO cells in the culture dish (diameter 35 mm) were incubated with 100 μCi ATB-[2- ^3H]BMPA (ref. 22) in 250 μl of PBS (10 mM), pH 7.4, for 1 min at 22 °C and then irradiated three times for 10 s each with a 625-W ultraviolet lamp (American Ultraviolet)²⁰. The cells were washed, homogenized and centrifuged as described in the legend to Fig. 1. The pellet was suspended in 1% SDS, 50 mM DTT and electrophoresed on 10% polyacrylamide gel. Gel lanes were sliced and radioactivity counted¹⁹. Experiments were carried out three times with similar results.

against this region (residues 478–492). This antibody did not react with the expressed 46K glucose transporter protein in clone DC37 (Fig. 1b, lane 3), but clearly identified the expressed 50K protein in clone B (lane 4). As previously observed²⁰, expression of the wild-type rabbit GLUT1 transporter resulted in a remarkable increase (~8-fold) in 2-deoxy-D-glucose uptake compared with control CHO cells (Fig. 1c). There was no significant increase in 2-deoxy-D-glucose uptake, however, in clone DC37 at low (0.1 mM) 2-deoxy-D-glucose concentrations. In addition, no significant increase in transport activity was observed in clone DC37 compared with control CHO cells at high 2-deoxy-D-glucose concentrations (10 mM) or with low (0.1 mM) and high (10 mM) 3-O-methyl-D-glucose concentrations (data not shown).

As cellular glucose uptake requires that the transporter be localized to the plasma membrane, we studied whether or not the normal and mutated glucose transporters were inserted into the plasma membrane. Glycoproteins at the surface of intact CHO cells were labelled by oxidation with galactose oxidase and subsequent reduction with borotritide²¹. Antibody against the C-terminal domain specifically immunoprecipitated the 50K protein from clone B but was unable to immunoprecipitate the 46K transporter in clone DC37 (Fig. 2a). By contrast, the labelled 46K protein was specifically immunoprecipitated from clone DC37 with antibody directed against the middle cytoplasmic loop region of the glucose transporter. Identical results

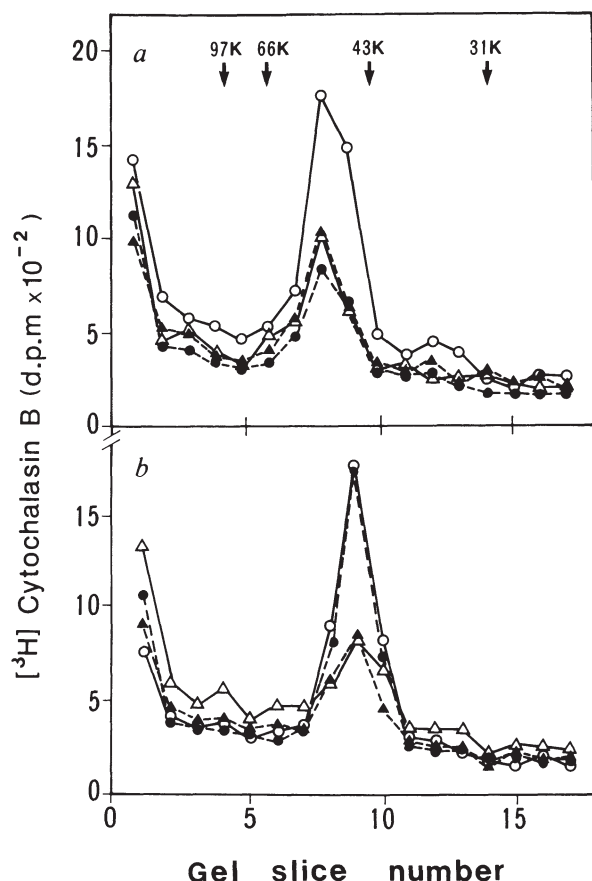


FIG. 3 Effect of D-glucose and side-specific D-glucose analogues on cytochalasin B-labelling of normal (a) and truncated (b) glucose transporter. The membranes from clone B and clone DC37 CHO cells were prepared as described in the legend to Fig. 1. Membranes (250 µg) were photoaffinity-labelled with 0.25 µM [³H]cytochalasin B in the presence of 100 mM D-sorbitol (○), D-glucose (△), 4,6-O-ethylidene-D-glucose (●), and *n*-propyl β-D-glucopyranoside (▲) and subjected to SDS-PAGE (10% acrylamide). Gel lanes were sliced and radioactivity determined. This experiment was repeated three times with similar results.

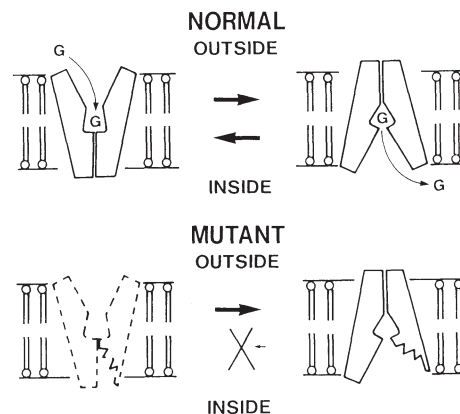


FIG. 4 Schematic representation of the 'alternating conformation' model in the normal (wild-type) and C-terminal deletion mutant of the glucose transporter. (One cannot exclude the possibility that the inner and outer glucose-binding site may be composed of completely different domains.)

were obtained when both the 50K and the 46K protein were immunoprecipitated with this antibody. Although the reason for the roughly twofold difference in immunoprecipitated labelled protein is not yet apparent, the small decrease in immunoprecipitation of the cell surface truncated glucose transporter cannot account for the fact that there is no significant increase in glucose transport activity (Fig. 1c). We also tried to label the glucose transporter with the photoaffinity-label probe 2-*N*-4(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3 bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA), which cannot enter the cells and binds only to the outer glucose-binding site of the transporter²². Very little labelled protein was found in clone DC37, whereas a large amount of labelled 50K protein was observed in clone B (Fig. 2b). These results, however, do not indicate that the mutant glucose transporter is not at the cell surface, as labelling with ATB-BMPA was hardly detectable in membranes isolated from clone DC37 (data not shown).

One explanation for these results might be that although the mutated glucose transporter is inserted into the membrane, it is not properly integrated and does not have a conformation that can bind glucose. However, the results of experiments with cytochalasin B strongly indicate that this is not the case. Photoaffinity labelling of the mutated glucose transporter in membranes from clone DC37 with [³H]cytochalasin B could be inhibited by D-glucose and also, the two transporters were labelled to a similar extent (Fig. 3a, b). The mutated and normal glucose transporters therefore have similar affinities for cytochalasin B and D-glucose. In addition, the mutant was recognized in cells by the anti-peptide antibody against the middle cytoplasmic loop region (residues 215–226) only when the plasma membrane of clone DC37 was permeabilized (data not shown). Thus, as in the normal transporter, the domain containing residues 215–226 is not exposed to the extracellular space in clone DC37.

As cytochalasin B binds to the inner face of the glucose transporter^{14,15} and ATB-BMPA binds to the outer²², these results indicate that only the inner glucose-binding site is available in the C-terminal deletion mutant. To address this issue, we took advantage of two glucose analogues, 4,6-O-ethylidene-D-glucose and *n*-propyl β-D-glucopyranoside, which preferentially bind to the outer and inner glucose-binding sites, respectively^{13,23}. The 4,6-O-ethylidene-D-glucose (100 mM) inhibited ~60% of the [³H]-cytochalasin B-labelling of the normal glucose transporter in the membranes of clone B through a conformational change induced by binding to the outer glucose-binding site, but it did not inhibit cytochalasin B labelling of the truncated transporter (Fig. 3a, b). These results are in marked contrast to the results with *n*-propyl β-D-glucopyranoside. This

compound inhibited [^3H]cytochalasin B labelling in both the normal and C-terminal truncated transporters by ~60%.

Tryptic digestion of inside-out human erythrocyte membrane vesicles has been reported to abolish hexose transport activity and reduce cytochalasin B binding¹⁵. Analysis of the trypsin-digested fragment of the human erythrocyte glucose transporter in conjunction with the sequence of the GLUT1 glucose transporter indicates that trypsin probably cuts out segments 213–269 and 457–492 (ref. 24). The latter cleavage is similar to the C-terminal deletion of the glucose transporter in clone DC37 in this study. Recently it was noted that labelling of trypsin-digested human erythrocyte glucose transporter with ATB-BMPA was very poor, less than 10% of that with the normal transporter²⁵, which is similar to the results obtained with clone DC37. Trypsin cleavage of the segment comprising residues 213–269 may be associated with a slight decrease in affinity for cytochalasin B.

Several studies using human erythrocytes expressing the HepG2 glucose transporter isoform have proposed an alternating conformation model for the mechanism of facilitated glucose transport^{11–17}. In this model the glucose transporter functions as a gated channel in which the glucose-binding site can exist on both the outer and inner side of the channel but cannot exist simultaneously at both; therefore, the glucose transporter should exist in either an outward- or an inward-facing form. Translocation of glucose across the membrane after binding to the transporter is thought to occur by a conformational change between these two forms. It has been suggested that the outer and inner glucose-binding sites are located in the highly conserved membrane-spanning segments M9, M10 and M11 (refs 22, 24).

The results presented here provide compelling evidence that the C-terminal truncated rabbit GLUT1 glucose transporter is properly integrated into the membranes in a fashion similar to the wild-type transporter, but that it has a conformation with only the inner glucose-binding site available. The C-terminal domain is not involved in binding of glucose or cytochalasin B to the inner binding site *per se*, but is required for formation of the outward-facing conformation (schematically represented in Fig. 4). The C-terminal truncated glucose transporter cannot alternate its conformation and is therefore locked into the inward-facing form. Thus, the outer gate of the channel is always closed in the mutated glucose transporter and glucose is unable to pass through. As facilitated glucose transporter isoforms share very similar structure^{1–10}, deletion of the C-terminal domain in other isoforms would also be expected markedly to reduce transport activity. It is possible that types of abnormal glucose metabolism in various individuals may result from this sort of mutation as this type of deletion is caused by introduction of a single point mutation for a stop codon. In addition, this sort of mutation may be of wide significance as any member of the superfamily of structurally related proteins²⁶ would also be expected to show functional impairment if such a mutation occurred spontaneously. □

Received 6 December 1989; accepted 26 March 1990.

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ACKNOWLEDGEMENTS. We thank Dr Geoffrey D. Holman for gifts of ATB-BMPA and *n*-propyl β -D-glucopyranoside and also for discussing his results before publication. This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science and Culture of Japan and a grant for diabetes research from the Ministry of Health and Welfare of Japan.

Small GTP-binding protein associated with Golgi cisternae

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EUKARYOTIC cells seem to use GTP hydrolysis to regulate vesicular traffic in exocytosis and endocytosis. The best evidence for this comes from studies on the yeast *Saccharomyces cerevisiae* that have identified two small Ras-related GTP-binding proteins, Sec4p and Ypt1p, which control distinct stages of the secretory pathway^{1–6}. In mammalian cells the effects of a non-hydrolysable GTP analogue, GTP- γ S, on different transport events have suggested that they also have proteins functionally related to yeast Sec4p and Ypt1p^{7–10}. The *rab* genes have recently been cloned and sequenced for rat and human and their proteins have highly conserved domains in common with Sec4p and Ypt1p (including a putative effector binding site). They are therefore good candidates for GTP-binding proteins involved in intracellular transport in mammalian cells^{11,12}. One of the Rab proteins (Rab1p) is the mammalian counterpart of Ypt1p (ref. 13). Here we report the localization of the protein Rab6p to the Golgi apparatus in several cell types. By immunolabelling and electron microscopy, Rab6p appears to be concentrated predominantly on the medial and trans cisternae and distributed over their entire surface.

The human *rab6* gene (*H-rab6*) encodes a GTP-binding protein (Rab6p) with a relative molecular mass (M_r) predicted to be 23,600 (23.6K) and having 26 and 36% amino-acid identity with Ypt1p and Sec4p, respectively¹². To determine the site of cellular function of this protein, we have raised polyclonal antibodies against Rab6p expressed in *Escherichia coli*. The antiserum obtained hybridizes to a band with an apparent M_r of 23K on a western blot of a total cell lysate prepared from human HEP2 cells (Fig. 1, lane 1) and from various human cell lines (data not shown). This antiserum is specific for Rab6p as it does not recognize other Rab proteins (Fig. 1, lanes 3–7) as well as Sec4p and p21^{ras} (data not shown). This indicates that the antibodies do not recognize the highly conserved GTP-binding and hydrolysis domains of the small GTP-binding proteins¹⁴. The antibodies detect a band on western blots of cell lysates of mouse (NIH 3T3; Fig. 1, lane 2), rat or hamster origin (data not shown) that has the same electrophoretic mobility as that detected in human cell lysates. This band probably represents the counterpart of the *H-rab6* gene product in rodent cells. We find that Rab6p is present in a wide variety of animal cells and tissues, but not *S. cerevisiae* (data not shown).

Most Rab6p was in the membrane fraction from HEP2 cells, but a small soluble fraction (~20%) was also consistently detectable (Fig. 2a). Treatment of membranes with either high pH, high salt or chaotropic agents such as urea did not affect the

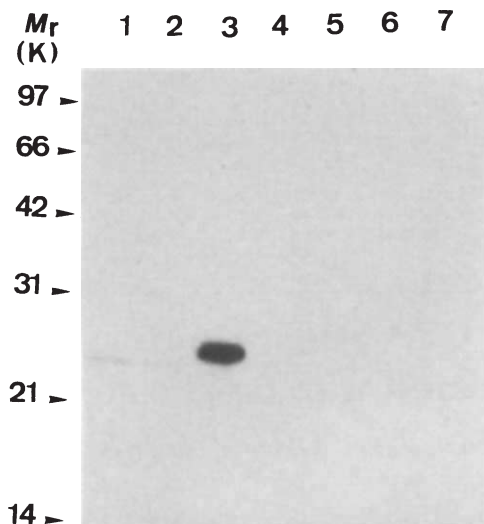


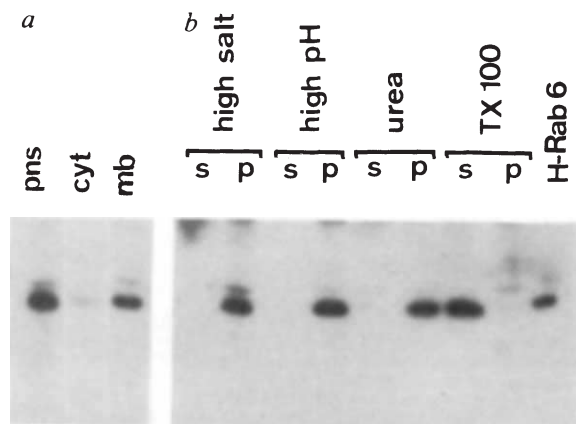
FIG. 1 Identification of Rab6p by immunoblotting. The following samples were subjected to SDS-PAGE, transferred to nitrocellulose, and hybridized with rabbit antiserum prepared against H-Rab6p: lane 1, 75 μ g total cell lysate from human epidermoid carcinoma (HEp2) cells; lane 2, 75 μ g total cell lysate from NIH 3T3 cells; lane 3, 20 ng purified H-Rab6p; lanes 4–7, 0.5 μ g purified H-Rab1p, 2p, 4p and 5p, respectively. The autoradiograph was exposed for 3 h at -80°C . The position of the molecular weight markers is shown on the left. Note that endogenous Rab6p migrates slightly faster than the H-Rab6p produced in *E. coli*, suggesting that there has been post-translational modification(s) of the protein specific for mammalian cells. METHODS. H-Rab proteins produced in *E. coli* were purified by column chromatography in a two-step procedure¹². H-Rab6p (50 μ g) was emulsified in Freund's complete adjuvant and injected into the popliteal lymph nodes of a rabbit (as described by Louvard *et al.*²⁴). To obtain total cell lysates, cells were collected from plastic tissue culture dishes with a rubber policeman, washed in PBS, and lysed with 0.3% SDS in 20 mM Tris-HCl, pH 8.8, 2 mM CaCl_2 containing 50 $\mu\text{g ml}^{-1}$ *Staphylococcus* nuclease, 0.1 mg ml^{-1} DNase I, 0.05 mg ml^{-1} RNase A and a mixture of protease inhibitors. Protein was quantitated by the method of Bradford²⁵. Proteins were separated on a 12% polyacrylamide-SDS gel²⁶ and transferred electrophoretically to nitrocellulose²⁷. Immunostaining was with ^{125}I -labelled Protein A (Amersham) to detect the primary antibodies bound to nitrocellulose² at a dilution of 1:200.

membrane association of Rab6p. But the protein could be solubilized by Triton X-100 (Fig. 2b). These results indicate that Rab6p is tightly bound to membranes by hydrophobic interactions, like Sec4p in yeast². Small GTP-binding proteins are synthesized as soluble proteins and undergo post-translational modification(s), allowing their attachment to cellular membranes¹⁵. Like Sec4p and Ypt1p, Rab6p has a cysteine residue¹² at its carboxy-terminal end which seems to be essential for membrane attachment^{3,16}. The membrane attachment of Ras proteins results from the addition of an isoprenyl chain to their COOH-terminal cysteine¹⁷. It should be pointed out that the proteins of the Rab/Sec4/Ypt1 family do not have the C-terminal consensus sequence CAAX required for the post-translational processing of Ras proteins¹⁷, which might suggest a different mechanism for their membrane attachment.

Visualized by immunofluorescence, affinity-purified anti-Rab6p antibodies stained a region next to the nucleus typical of the Golgi complex in all the cells lines studied, including

NIH 3T3 (Fig. 3a), and normal rat kidney (NRK) cells (Fig. 3c). Double labelling of NRK cells showed that the antibodies labelled the same structure as the monoclonal antibody CTR 433, which reacts with the medial cisternae of the Golgi apparatus¹⁸ (Figs. 3c and d). We used immunoperoxidase staining and electron microscopy to visualize Rab6p in the Golgi region of NRK cells at higher resolution. The peroxidase reaction product was localized predominantly to the cytoplasmic surface of Golgi cisternae (Fig. 4). The staining was usually uniformly distributed over the entire surface of the cisternae, although in some cases a more patchy distribution was observed (Fig. 4d). In all the Golgi complexes examined the staining appeared to be polarized and was absent from one side of the cisternal stacks. Although it is difficult to assign the polarity of the stacks in cultured cells, this side was identified as the *cis* face through its typical fenestrated morphology¹⁹ and by staining of NRK cells with antibodies against a 58K membrane protein (p58), a component of the *cis* Golgi cisternae²⁰ (Fig. 4b, inset).

FIG. 2 Rab6p is mostly membrane-bound and is tightly associated with membranes. The following samples were analysed by immunoblotting with anti-H-Rab6p antibodies: a, from left to right; pns, 50 μ g post-nuclear supernatant from HEp2 cells; cyt (cytosol), 40 μ g high-speed supernatant; mb (membranes), 10 μ g high-speed pellet. The band seen above Rab6p in pns and mb fractions was also detected with the pre-immune serum (data not shown). b, Aliquots (10 μ g) of the membrane fraction were treated with 1 M NaCl (high salt), 0.2 M Na_2CO_3 (high pH), 6 M urea and 1% Triton X-100 respectively. Only Triton X-100 released Rab6p from the membrane (P) to the supernatant fraction (S). In the right-hand lane, the sample was 10 ng of purified H-Rab6p. The autoradiograph was exposed for 24 h at -80°C . METHODS. HEp2 cells were collected by scraping into PBS containing 1 mM phenyl methane sulphonyl fluoride (PMSF). After washing, cells were homogenized on ice using 10 strokes of a ball-bearing homogenizer (Berni-tech engineering) in buffer A (10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM MgCl_2 , 5 mM CaCl_2 and a mixture of protease inhibitors). After pelleting nuclei (at 600g for 10 min), the post-nuclear supernatant (pns) was centrifuged at 100,000g for 1 h to generate a cytosol fraction (cyt) and a crude membrane fraction (mb). In the experiment shown in a, aliquots of pns, cytosol and membrane fractions were boiled in SDS and processed for immunoblotting. The amount of protein loaded onto the gel was standardized to take into account the distribution of total proteins between cytosolic and membrane fractions. The quantitation of the amount of Rab6p in the different fractions was as described². In the experiment shown in b, 200 μ g of the membrane fractions were resuspended in 1 M NaCl, 6 M urea or 1% Triton X-100 in buffer A, or 0.2 M sodium carbonate (pH 11) in water. After incubation on ice for 30 min, the samples were centrifuged for 1 h at 100,000g. The supernatants and pellets (resuspended in the same volume of buffer A) were boiled in SDS and run on a 12% SDS-PAGE gel.



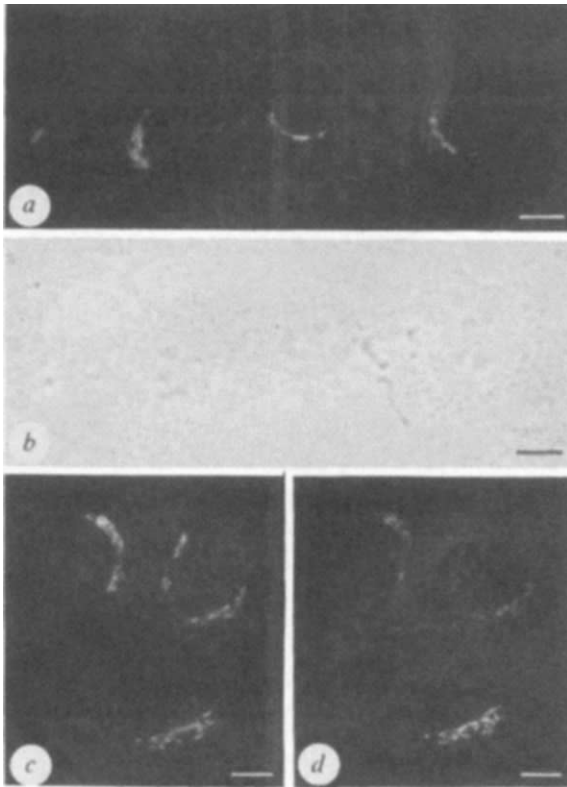


FIG. 3 Localization of Rab6p by immunofluorescence. *a*, staining of Golgi-like structures near the nucleus in NIH 3T3 cells with anti-H-Rab6p antibodies. *b*, A phase contrast view of the same field as seen in *a*. *c* and *d*, Double labelling of normal rat kidney (NRK) cells with anti-H-Rab6p antibodies (*c*) and a monoclonal antibody (CTR 433) against the Golgi complex (*d*). Note the almost complete overlap of the staining patterns obtained with the two antibodies. The bars correspond to 10 μ m.

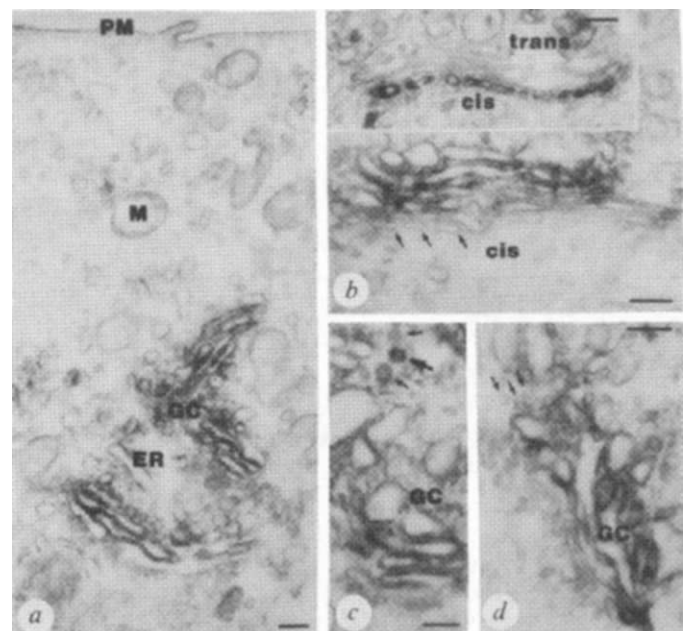
METHODS. The antibodies were affinity-purified by binding to H-Rab6p transferred to nitrocellulose according to Talian *et al.*²⁸. Cells were grown for two days on glass coverslips and fixed in 4% paraformaldehyde, washed in PBS, quenched for 15 min with 50 mM NH₄Cl in PBS, and then permeabilized for 15 min in PBS containing 0.2% BSA and 0.05% saponin (PBS-BSA-saponin). All subsequent incubations with antibodies and washes were in this buffer. Cells were incubated with affinity-purified anti-H-Rab6p antibodies for 1 h then with rhodamine-conjugated goat anti-rabbit antibodies (Biosys) for 1 h. In double-labelling experiments, cells were first incubated with a mixture of anti-H-Rab6p antibodies and mouse monoclonal CTR 433 antibodies, then with a mixture of rhodamine-conjugated goat anti-rabbit antibodies and fluorescein-conjugated sheep anti-mouse antibodies (Amersham or Biosys). Cells were mounted and examined in a Leitz Dialux microscope and photographs taken with Kodak Tri-X-Pan (400 ASA) film.

These findings suggest that Rab6p is predominantly associated with the cytoplasmic surface of medial and *trans* Golgi cisternae. The small vesicles in the periphery of the Golgi complex were occasionally seen to contain this antigen (Figs 4*c* and *d*).

Small GTP-binding proteins may use GTP hydrolysis to trigger a conformational switch promoting unidirectional movement of transport vesicles from a donor compartment to an acceptor

compartment (discussed by Bourne²¹). This model predicts that the GTP-binding proteins should be present on both the donor and acceptor compartments and in both soluble and membrane-bound forms. Therefore, the characteristics of Rab6p make it a candidate for a GTP-binding protein controlling a transport event at the level of medial and *trans* Golgi cisternae. The traffic of transport vesicles (containing secretory and plasma

FIG. 4 Localization of Rab6p in NRK cells by immunoperoxidase electron microscopy. *a*, The peroxidase reaction product is seen in the Golgi complex (GC); the plasma membrane (PM) and intracellular organelles such as the endoplasmic reticulum (ER) and mitochondria (M) are unlabelled. *b*, In the Golgi complex the labelling is seen predominantly on the cytoplasmic aspect of medial or *trans* cisternae, whereas one or two cisternae at the *cis* side are devoid of label. The small arrows indicate the typical fenestrations of the *cis*-most cisterna with antibodies against a 58K membrane protein (p58). *c* and *d*, The small coated or uncoated vesicles present in the periphery of the Golgi complex (small arrows) are typically not labelled with anti-H-Rab6p antibodies. The thick arrow in *c*, however, indicates one such labelled vesicle which seems to be coated. The scale bars correspond to 0.2 μ m. **METHODS.** NRK cells were grown to ~70% confluency on plastic tissue-culture dishes. Medium was removed and cells were fixed for 2–4 h at room temperature in paraformaldehyde-lysine-periodate fixative²⁹. The pre-embedding immunoperoxidase procedure has been described^{20,30}. Briefly, the cells permeabilized in PBS-BSA-saponin (see legend to Fig. 3) were then incubated with affinity-purified anti-H-Rab6p antibodies or polyclonal rabbit antibodies prepared against p58 purified from rat pancreas (J. S. and K. Svensson, manuscript in preparation) diluted in PBS-BSA-saponin. After washing and incubation with peroxidase-conjugated (Fab)₂ fragments of goat anti-rabbit IgG (Immunotech) in PBS-BSA-saponin, the cells were post-fixed in 1.5% glutaraldehyde. The peroxidase reaction using diaminobenzidine as substrate was carried out on ice. Thereafter the cells were fixed in reduced osmium, dehydrated in ethanol, scraped from the dishes, pelleted, and embedded in Spurr. Thin sections were post-stained with lead citrate and examined and photographed in a JEOL 100CX electron microscope operated at 60 kV.



membrane proteins) between the cisternae is thought to occur mainly at the dilated rims of the Golgi stacks^{22,23}. If Rab6p were involved in this process, it would be expected to be more concentrated at the edges of the cisternae. But the exact topology of intra-Golgi traffic has been very difficult to examine *in vivo* and there is no strong evidence to support this model. Alternatively, Rab6p could be involved in an as-yet-unknown transport event between the Golgi stacks.

Finally, the observation of the polarized distribution of Rab6p in the Golgi apparatus is of interest. This suggests that another GTP-binding protein may act in intra-Golgi transport before Rab6p. Such a protein could be Rab1p, the mammalian counterpart of yeast Ypt1p (refs 5, 12, 13). Our results support the hypothesis that several small GTP-binding proteins localize to different intracellular compartments and have a pivotal role in maintaining the orderly flow of vesicular traffic in mammalian cells. □

Received 25 January; accepted 27 March 1990.

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ACKNOWLEDGEMENTS. We thank Dr Gerard Buttin for his support; Dr Johan Thyberg for discussion and for electron microscope facilities; Dr Michel Bornens for the monoclonal antibody CTR 433; and Drs P. Boquet, A. Dautry-Varsat, D. Louvard and M. McCaffrey for critically reading the manuscript. This work was supported in part by the Université P. et M. Curie and by the Ligue Nationale Française contre le Cancer.

CORRECTION

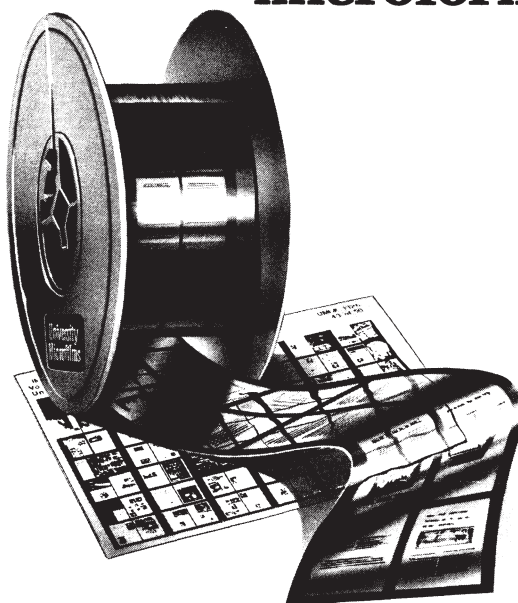
Role of sub-micrometre particles in the ocean

Isao Koike, Shigemitsu Hara, Kazuki Terauchi & Kazuhiro Kogure

Nature **345**, 242-244 (1990)

THE listing of the authors in this paper in the 17 May issue may cause some confusion to indexers. The above listing is correct, though in the printed version the first author was given as Koike Isao, following the Japanese convention of placing surnames first. The listing on the contents page is correct. □

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Flow cytometry in toxicity analysis

C.E Dallas and D.L. Evans

The well-established clinical research tool of flow cytometry has now been applied to the detection of toxic injury to the cells of organisms exposed to environmental contaminants.

THE need for rapid, accurate and relatively inexpensive tests for detecting various forms of toxicity produced by exposure to environmental contaminants has become increasingly important. The contamination of the workplace, drinking water sources, and indoor air environments has led to an increased risk to human public health. Many environmental agents are now known to cause significant toxicity to wildlife and other important organisms of ecosystems. The wide spectrum of chemical and radioactive contaminants now being detected in various environments include many agents that are known to be clastogenic and carcinogenic.

Testing systems for cytogenetic analysis have therefore become an important part of the risk-assessment process for environmental clastogens and carcinogens. Detection of chromosomal changes or aberrations in organisms exposed to these agents by microscopic scoring has been used widely in the past as a common means of cytogenetic screening. However, the cost of this approach is quite significant and there is some variability in the results obtained due to the level of expertise and accuracy of the personnel involved. Also, time and cost constraints usually result in data generated from enumerating only 100–200 cells, when 10,000 cells are considered representative¹. To address these cytogenetic testing needs, flow cytometric techniques have now been applied to uses in the monitoring and evaluation of environmental contaminants.

Flow cytometry approach

Over the past 20 years, instruments for flow cytometry have been developed to analyse cells relative to a variety of biochemical parameters. In particular, the relative quantities of DNA and the distribution of nucleic acids in various stages of the cell cycle have been measured with these devices. One striking advantage of this approach is that a large number of cells can be analysed rapidly. Typically, 10,000 cells can be measured in only a few seconds². Most current approaches in flow cytometry use the exposure of properly stained cells to a collimated, focused beam of light so that the physical properties of each cell, such as size and cytoplasmic granularity, can be measured (Fig. 1). This, combined with the ability to detect fluorescent light (at different wave-

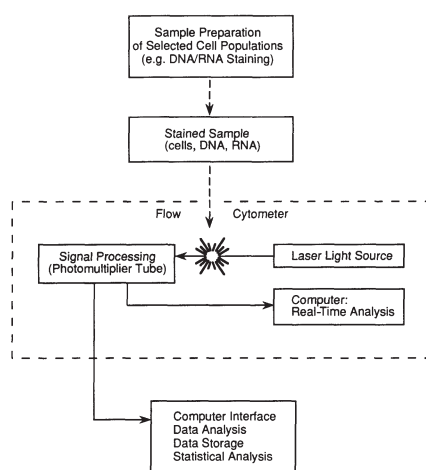


FIG. 1 Schematic diagram of the basic flow cytometric methodological approach for a flow cytometer using a laser as a light source.

lengths) and light emitted by DNA and/or RNA intercalating dyes, makes flow cytometry an extremely useful clinical and research tool. The influence of drugs on cell-cycle progression is one such application of flow cytometry that has widespread applications in studies of immunocytotoxicity and cytotoxicology.

Applications to toxicology

The potential for drugs and other possible cytogenetic agents to induce cytotoxic or genotoxic cellular effects has been measured using flow cytometry. DNA-specific fluorescent staining has been used for cytogenetic analysis of potentially toxic drugs by measuring changes in cellular DNA content and altered cell-cycle progression^{3,4}. The different phases of the cell cycle, such as G_0/G_1 , S, and $G_2 + M$, are accurately quantified by flow cytometry (Fig. 2). These methods have also been

shown to be useful in measurements of drug-induced ploidy changes related to effects on the cell cycle^{5,6}.

Studies using flow cytometry to detect clastogenic effects have shown that an increased dispersion of the cellular DNA content can be produced in a proliferating cell population as a result of clastogen-induced structural chromosome aberrations. In dividing cells, this leads to an unequal distribution of DNA in the daughter cells⁷. Thus, one biomarker response for clastogens using flow cytometry is a broadening of the G_1 peak in cells exposed to the cytotoxic agent. At doses that produce broken chromosomes in about 80 per cent of cells exposed to a clastogen, flow cytometric analysis reveals a broadening of the entire G_1 peak⁸. Relatively low doses of certain clastogens have resulted in a broadening of the base of the G_1 peak¹. Another significant advantage of flow cytometric analyses is the degree to which cytogenetic effects can then be quantified and subjected to more rigorous statistical analyses. Increases in the coefficient of variation (c.v.) in the G_1 peak have been observed in cells treated with a variety of mutagens, thus demonstrating the usefulness of flow cytometry as an *in vivo* mutagenicity test⁹.

Flow cytometry has been used to determine RNA content and distribution in cells exposed to mutagens and carcinogens¹⁰. This type of analysis is now considered to be an accurate assay system for determining chemical toxicity². Flow cytometry is also considered to be the technique of choice for quick and accurate detection of aneuploidy in cell populations exposed to cytotoxic agents. Differences in DNA of less than one per cent of the human genome can accurately be detected using flow cytometry. This sug-

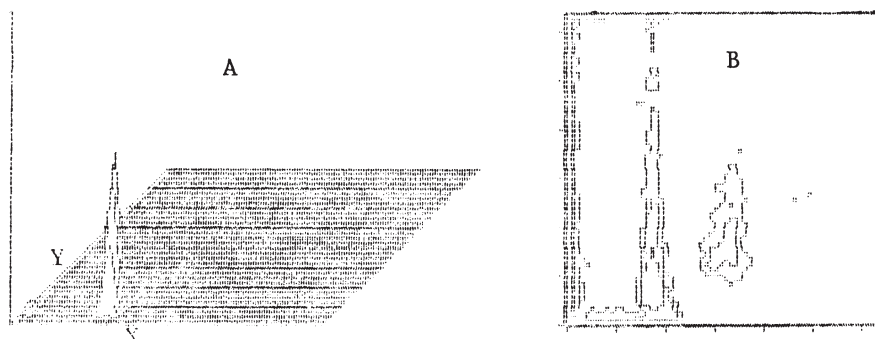


FIG. 2 DNA histograms of duck red blood cell DNA. a, three-dimensional plot of the G_0/G_1 phase; b, two-parameter analysis showing distinct plots for the G_0/G_1 and $G_2 + M$ phases.

gests that aneuploids involving the smallest human chromosomes can be observed cytometrically¹. Indeed, it has been suggested that monitoring aneuploids in human leukaemia stemlines by flow cytometry may be a useful diagnostic tool⁴.

Sentinel animal biomarkers

One recent application of flow cytometry in toxicity testing has been the monitoring of 'sentinel' animals in the vicinity of significant environmental concentrations of potent clastogens and mutagens, such as in or around toxic waste facilities or contaminated bodies of water. The sentinel animals (that is, their DNA content) may serve as an indicator of environmental contamination for relative risk assessments for man and the ecosystem.

Aneuploidy and increases in G_0/G_1 c.v. have been detected using flow cytometry in several wildlife species exposed to a variety of environmental cytogenetic agents. Spleen cells from rodents exposed at a waste site to complex mixtures of chemical mutagens contained aneuploid DNA¹¹. Statistically significant increases in G_0/G_1 c.v. and aneuploids were reported in red blood cells taken at one point in time from turtles on a radionuclide-contaminated pond¹². The time course of internal radionuclide uptake has been monitored simultaneously with flow cytometric measurements of red blood cells over a one-year period in previously uncontaminated mallards on the same pond, and aneuploids were observed after long-term exposure¹³. The researchers involved in these studies have concluded that flow cytometric analysis of cell populations from sentinel animals in and around contaminated sites can be a useful approach to monitoring the prevalence and toxicity of environmental contaminants. □

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Tissue culture tips

A benchtop bioreactor and insect cell culture media are among the exhibits at next week's Tissue Culture Association meeting in Houston, Texas, which covers plant, vertebrate and invertebrate cell, tissue and organ culture.

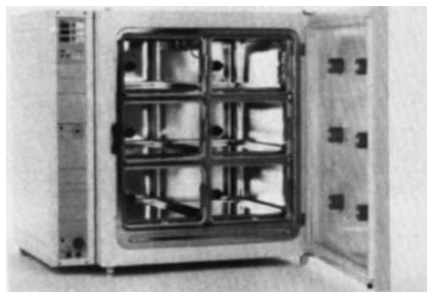
PRODUCING gram quantities of mammalian cell products has never been simpler, says New Brunswick Scientific, when using its CellTronics HF-100 **benchtop hollow-fibre bioreactor system** (Reader Service No. 101). The CellTronics HF-100 consists of a presterilized bioreactor contained within a temperature-controlled incubator. The built-in fluid transport



NBS's benchtop bioreactor system.

system provides automatic circulation of oxygenated medium and removal of waste products. Other features of this fully scalable system include reverse-flow cycling, which results in high yields of secreted proteins; reduced use of serum and growth factors; and microprocessor control of all critical process parameters via a touch-sensitive keypad. Visit NBS in booth 104.

The BB 6220 **gassed incubator** from Heraeus is designed to provide digital control of preselected environmental parameters: temperature, humidity, CO_2 and oxygen partial pressure (Reader Service No. 102). The BB 6220 incubator has a chamber volume of 220 litres: the inner chamber is equipped with six gas-tight glass doors instead of a single door. This, says Heraeus, reduces the possibility of contamination during loading and reduces interference with the chamber's controlled atmosphere. Other features include a switchable high/low humidity



Heraeus' six-door gassed incubator.

system, a fully automatic calibration system for oxygen and CO_2 , automatic start-up, and a microprocessor-based monitoring and control system. See the full lineup of Heraeus hardware in booth 603.

Media alert

Sigma Cell Culture, exhibiting in booth 401, is offering Heartline **fetal bovine serum (FBS) and horse serum**, which is designed to meet exacting standards for endotoxin, haemoglobin and IgG levels (Reader Service No. 103). Available in 50-, 100- and 500-ml quantities, Heartline FBS is screened for mycoplasma, virus and bacteriophage contamination: endotoxin levels are ≤ 0.5 ng ml⁻¹. Sigma's horse serum is obtained from donor herds and packaged in 100- and 500-ml quantities. The donor herd is tested for tuberculosis, glanders, pyroplasmiasis, equine infectious anaemia (EIA), dourine and brucellosis. The horse serum is screened for EIA.

See booth 105 for Life Technologies' selection of serum-free and serum supplemented **insect cell culture media** (Reader Service No. 104). The range includes SF-900 serum-free insect medium for suspension and monolayer insect cell culture methods, Grace's insect medium supplemented with lactalbumin hydrolysate and yeastolate, TC-100 medium for insect cell lines used as hosts in the study of virus replication and, lastly, IPL-41 medium for optimized growth of Sf9 cells for the baculovirus expression system. □

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